Diversity-function relationships in natural, applied, and engineered microbial ecosystems

Diversity-Function Relationships in Natural, Applied and Engineered Microbial Ecosystems.

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Contents

Abstract

The connection between ecosystem function and taxonomic diversity has been of interest and relevance to macroecologists for decades. After many years of lagging behind due to the difficulty of assigning both taxonomy and function to poorly-distinguishable microscopic cells, microbial ecology now has access to a suite of powerful molecular tools which allow its practitioners to generate data relating to diversity and function of a microbial community on an unprecedented scale. Instead, the problem facing today's microbial ecologists is coupling the ease of generation of these datasets with the formulation and testing of workable hypotheses relating the diversity and function of environmental, host-associated and engineered microbial communities.

Here we review the current state of knowledge regarding the links between taxonomic alpha- and beta-diversity and ecosystem function, comparing our knowledge in this area to that obtained by macroecologists who use more traditional techniques. We consider the methodologies that can be applied to study these properties, and how successful they are at linking function to diversity, using examples from the study of model microbial ecosystems, methanogenic bioreactors (anaerobic digesters) and host-associated microbiota. Finally, we

assess ways in which our newly-acquired understanding might be used to manipulate diversity in ecosystems of interest in order to improve function for the benefit of us or the environment in general through the provision of ecosystem services.

1. INTRODUCTION

Microorganisms make up the greatest majority of life the biosphere and survive in diverse and complex communities, where they evolve to act as drivers of ecosystem function (Falkowski, Fenchel, & Delong, 2008; Widder et al., 2016). Within these communities, microorganisms exist in relationships that can be collaborative or competitive, and species present in a particular environment vary widely across microbial taxa (Justice et al., 2017). Microbial populations play key roles in driving nutrient cycling in the environment, maintaining soil structure and forming symbiotic relationships with higher organisms (Kowalski et al., 2015). The global importance of microbial communities cannot be overstated in regard to maintaining human, animal and plant health; microorganisms enhance the mammalian metabolic repertoire, train the cells of the immune system, and influence plant phenotypes. In addition, they give rise to sustainably produced renewables in biotechnology.

Previously, the importance of microbial ecology could not be fully recognized as studying microbial populations proved challenging for a number of reasons. Initially, only traditional cultivation studies were used to characterize microbial populations, but the ability to culture microorganisms is hampered since the exact nutritional requirements (and potential evolutionary synergistic partnerships) for the majority of microorganisms are not known. This uncultured majority (or the "microbial dark matter" of the biosphere) left a gap in the knowledge regarding the true extent of microbial biodiversity and function (Rinke et al., 2013).

Current strides towards a more sustainable society based on a productive bio-economy rely on many biotechnologies driven by the action of microorganisms (Staffas, Gustavsson, & McCormick, 2013). However, the interplay between species within microbial communities is what *drives* such processes and ensures their success. Therefore, it is essential that most community members are recognized and their function elucidated. This has not been able to occur previously - and so the field of microbial ecology has lagged behind that of macroecology - and often, microbially-driven biotechnologies have been prone to failure as the microbial consortia have not been well characterized, and therefore not optimized for a particular function (Jiang, Guo, & Zhang, 2016).

With the development of molecular biology techniques, combined with traditional microbiological techniques, has microbial ecology has become better understood. Importantly, there has been a move away from cultivation studies as the limitations of culture-dependent approaches have become clear (Hilton et al., 2016). This helps prevent rediscovery of known population members and provides a more unbiased approach to examining microbial biodiversity. Newer and more powerful molecular techniques have allowed microbial ecologists to access a wealth of data regarding the microorganisms within particular habitats, allowing the structure and function of complex microbial consortia to be better understood. The ease of access to large datasets has given rise to a pressing issue: how do microbial ecologists use the community data to address reproducible and robust hypotheses, and how do they link community composition to function within a particular system?

How these communities assemble and function is only beginning to become clear (Thompson et al., 2017). For example, it is now clear that low abundance microorganisms can act as critical functional members of the community (Kleindienst et al., 2016). This is evident from studies such as that by Wilheim et al. (2014), which show that the low abundance taxa present in microbial populations (such as biofilms) can play crucial roles, and their absence can cause the microbial population to become less resilient or to fail. Some low abundance taxa can flourish under specific environmental conditions ("conditionally rare" organisms) and drive the success of the whole community (Kleindienst et al., 2016; Shade et al., 2014). It has also been noted that dormant microorganisms can play key roles in the maintenance and assembly of successful communities (Jones & Lennon, 2010).

It is evident that the field of applied microbiome studies will continue to expand (Marchesi & Ravel, 2015). It should also be noted that disruption of microbial populations can lead to disease states in their associated hosts. These disrupted communities also require further study to enhance host health, and assist in the prevention and management of disease.

The remainder of this chapter will discuss methodologies available to microbial ecologists in order to carry out robust studies on microbially-driven systems, and then will focus on linking the alpha- and beta-diversity of microbial populations to ecosystem function in well-understood systems (including model microbial communities, bioreactor populations, and host-

associated microbiotas). Finally, we will discuss how manipulation of microbial communities can be utilised in order to test ecological relationships and perform selective functions.

1.1 Biodiversity in Macro- and Microbial Ecology

The term "biodiversity" (conjuncture of the previously used "biological diversity") was first used to describe all living organisms within the biosphere in 1985 by Walter G. Rosen (Harper & Hawksworth, 1994). Many countries have developed policies that attempt to engage in efforts to monitor biological diversity across a range of scales and timeframes (Yoccoz, Nichols, & Boulinier, 2001). However, as stated by Krebs (1991): "monitoring of populations is politically attractive but ecologically banal unless it is coupled with experimental work to understand the mechanisms behind system changes". In other words, diversity indices hold little value if not linked through hypothesis-driven approaches (see Box 1).

Many more ecological studies have focused on the scale of plants and animals (macroecology) compared to microorganisms (microbial ecology), as plants and animals can be linked with ease to land management practices and conservation efforts in the face of over-exploitation of natural capital (Rossi et al., 2017; Tófoli et al., 2017). The field of microbial ecology has lagged behind studies in macroecology due to challenges in assessing the overall diversity of microbial species present in the biosphere. An ecological metaphor for comparing micro- with macroecology is discussed by Madsen (2008), which is that cultured microorganisms (the known one percent) are similar to domesticated animals, while the dark matter microorganisms are like wild animals; unmanaged and elusive.

Traditionally, macroecologists have used counting/observation methods to study wild populations, but more recently, they have also have developed novel techniques such as satellite imaging and other non-invasive approaches to access biodiversity in attempts to close the gap between data obtained and theory (Keith et al., 2012; Pimm et al., 2015). Nevertheless, these macroecological techniques cannot be applied to microorganisms given their small size and ubiquitous nature in terrestrial and aquatic environments; as it stands, it is predicted that Earth is home to one trillion (10^{12}) microbial species (Locey & Lennon, 2016). It is therefore evident that microbial ecology needs to rely on multiple approaches to detail life at the

microscopic level, integrating the use of traditional ecological theory (e.g. Box 1) with novel genetic approaches.

1.2 Diversity: Who is There, and What are They Doing?

Pioneering work by Woese in the 1970s showed that microorganisms could be classified by characterization of a conserved gene, the ribosomal RNA (rRNA) gene, which led to the discovery of the third domain of life: the *Archaea* (Woese & Fox, 1977). The small subunit or 16S rRNA gene later became the target of choice for molecular characterization of microbial diversity (e.g. fingerprinting methods and cloning followed by Sanger sequencing), which resulted in numerous metataxonomic studies on a wide range on environments (i.e. who is there? Table 1). These studies revealed a surprising amount of diversity, even in environments considered too hostile or extreme for life to thrive (Sonne-Hansen, Mathrani, & Ahring, 1993; Skirnisdottir et al., 2000).

In the last decade, next-generation DNA sequencing (NGS) technologies have re-energized microbial ecology by allowing microbial communities to be characterized to unprecedented depths. Excitingly, this high-throughput approach allows elucidation of members of the rare biosphere that previous clone-based methods were unable to access, and that culture-based methods accessed only selectively (Fig. 1). With improvements in sequencing technologies and bioinformatics pipelines, gaining greater numbers of sequencing reads (typically obtaining thousands to millions of reads per sample) is now possible at a lower cost than with previous technologies. A range of platforms are available, with outputs varying in read length and sequencing depth; often, one comes at the expense of the other (Goodwin, McPherson, & McCombie, 2016).

Increasingly, NGS technologies have facilitated metagenomic studies (defined as studies of all the genomes in a given environment). Construction of metagenomes has given a greater insight into microbial diversity and helped to expand the tree of life by documenting members of candidate phyla, further tapping into the microbial dark matter (Parks et al., 2017). For example, metagenomic analysis of deep-sea sediments has allowed the discovery of a novel phylum of *Archaea*, termed the *Lokiarchaeota*, which have been suggested to be monophyletic with the ancestral eukaryotes (Spang et al., 2015). However, these studies go beyond simple meta-taxonomic studies of microbial diversity; they aim to determine the genetic potential of

a community, and therefore possible community functions (i.e. what can they do? Table 1). A limitation of such approaches is that, with current sequencing and bioinformatics methods, relatively complete genomes can only be assembled from the more abundant members of a community, unless initial culture-independent isolation such as single-cell sorting followed by whole-genome amplification (Rinke et al., 2013) is applied first. Another approach is to perform functional gene assays that assess members of a functional group by targeting functional genes (for example using the *nos*Z gene to investigate microbes involved in the nitrogen cycle (Henry et al., 2006).

Metatranscriptomics, metaproteomics and metabolomics provide further information on community function, but in real time (i.e. what are they doing? Table 1). Metatranscriptomics detects both phylogenetic and metabolic RNA markers, therefore simultaneously characterizing both microbial community composition and metabolically active members of the community (Jiang et al., 2016). The advantage of this approach is that it only detects live cells, whereas the aforementioned DNA-based methods do not distinguish between live and dead cells. Metaproteomics approaches investigate the proteins that are expressed by the community either over time and/or in association with a specific treatment (Zhang et al., 2017b). Similarly, (meta)metabolomics approaches investigate organic metabolites made by living cells to characterize cell-environment and cell-cell interactions (Yang et al., 2011). A graphical summary of molecular methods used in the characterization of microbial communities is shown in Fig. 2.

1.3 Defining Microbial Species and the Operational Taxonomic Unit (OTU)

Defining a microbial species is problematic. The approaches used by macroecologists (morphological traits, interspecies behavior and the ability to produce fertile offspring) cannot be applied to organisms classified within the bacterial and archaeal domains (Fraser et al., 2009). Despite being routinely used in the 20th century to assess the concept of a microbial species, microbial morphology and metabolic preferences, combined with microscopy are insufficient to distinguish between microbial species (Staley, 2006). The issue remains complex due to the fast rate of microbial evolution and adaptation, as well as the subsequent emergence of new strains of different species. Furthermore, Venton (2017) discusses how the microbial species debate remains contentious and hypothesizes that microorganisms (much

like higher organisms) speciate depending on a specific trigger (e.g evolutionary advantage/geographical isolation). However, the concept of how microbial species arise is made more difficult due to microbial gene-flow processes [such as horizontal gene transfer (HGT)], and Bobay and Ochman (2017) argue that barriers to homologous recombination define biological species within the prokaryotes. It is clear that the debate into the true definition of a microbial species is still on-going; however, microbial ecologists have several working definitions to assign taxonomy to the species level.

Traditionally, if a microorganism had been successfully cultured from an environment of interest, DNA-DNA hybridization could be used to assess whether two microorganisms were the same species. However, this technique is not without limitations and can only be applied following successful isolation of microorganisms into axenic states. Given the advances in NGS and –omics approaches, definitions of microbial species have relied more heavily on the use of the operational taxonomic unit (OTU) to gain a more in-depth understanding of microbial biodiversity, particularly in the rare biosphere (Huse et al., 2010).

As the concept of microbial speciation remains controversial, using the OTU as a defined cluster of 16S small-subunit (SSU) rRNA sequences which have ≥97% identity has proved advantageous in characterizing bacterial and archaeal communities through established bioinformatics pipelines (Schmidt, Matias Rodrigues, & von Mering, 2014). The 97% cut-off used to define an OTU is a result of a study that demonstrated that most species had 97% 16S rRNA sequence similarity (Konstantinidis & Tiedje, 2005) Alternative gene targets for fungal communities (the internal transcribed spacer (ITS) region) and other eukaryotes (SSU 18S rRNA) can also provide characterization of such communities, and OTUs can be defined similarly for these markers (Callahan et al., 2016). The OTU has proved to be a powerful means to categorize microorganisms (Schmidt, Matias Rodrigues, & von Mering, 2014). Commonly used bioinformatics pipelines such as QIIME (Quantitative Insights into Microbial Ecology) provide robust and effective means to convert microbial sequence data into traditional ecological data and allow analysis to occur which was not achievable using lower throughput sequencing technologies (Caporaso et al., 2010).

However, despite widespread utilization of the OTU as a means of reflecting “true” microbial taxa, it is not without limitations. Use of an arbitrary 97% identity cut-off can both fail to take

account of genuine low/fine scale variation between sequences or yield artefactual OTUs which are due to technical errors in PCR or sequencing (Callahan et al., 2016), despite steps taken to filter likely erroneous sequences from data-sets (Siegwald et al., 2017). If OTUs are defined *de novo* (within only the dataset) then inter-study comparisons cannot be made without full re-clustering, while attempts to overcome this issue by clustering study sequences with reference database sequences exclude analysis of sequences not represented in the database at the time of analysis (Callahan, McMurdie, & Holmes, 2017). Newer methods such as the Divisive Amplicon Denoising Algorithm [DADA2; (Callahan et al., 2016)] instead define exact Amplicon Sequence Variants (ASVs), which simultaneously overcome both of these problems. ASV methods such as DADA2 and minimum entropy composition [MED; (Eren et al., 2015)] therefore have the potential to both increase the accuracy and utility of individual metataxonomic studies and to future-proof those studies against database revisions and the subsequent acquisition of additional data. DADA2 has allowed the identification of specific biogeography within the human archaeota (Koskinen et al., 2017) and of novel *Lactobacillus* diversity in the vaginal microbiota (Callahan et al., 2016), while the MED-based oligotyping method reveals seasonal variation in oceanic *Pelagibacter* species (Eren et al., 2013). The use of finer scale differentiation between sequences aids in elucidating the ecology of samples where there may be community members which are poorly characterized in databases and ASVs may provide a more accurate picture of diversity (Callahan, McMurdie, & Holmes, 2017).

Lastly, it should be stressed than assigning taxonomy to sequences relies on the use of well-maintained and high quality taxonomic databases which contain reference sequences which allow allocation of taxonomy. Several databases are routinely applied to microbiome studies (e.g SILVA, Greengenes, RDP), which rank sequences or "nodes" to their taxonomic assignment (Balvociute and Huson 2017). The revision of these databases as new taxonomic information emerges is crucial to maximising the information gained from metataxonomic studies, and the database-independence of ASV-based analysis removes the need for full re-analysis when such revisions occur (Callahan, McMurdie, & Holmes 2017). Another important caveat to such studies is that they generally only generate relative abundance information for the microbial taxa identified (Widder et al., 2016); recent attempts to overcome this limitation by correcting for total microbial load in the samples compared suggest that this can dramatically alter the patterns of inter-sample relationships detected (Vandeputte et al., 2017).

1.4 Defining Diversity

Measuring diversity is critical for our understanding of the structure of biologically relevant communities and how shifts in community structure can occur over time. There are several approaches to do so (as reviewed by Lozupone and Knight (2008). There are two key indices of diversity that are fundamental in large-scale and microbial scale ecological studies; alpha (α) and beta (β) diversity. Alpha diversity refers to the amount of variation within a single sample (either the richness of the sample, and/or the evenness of species distribution). For the measurement of microbial alpha diversity, Shannon and Simpson diversity can be estimated robustly from metataxonomic data, unlike some other measures (Haegeman et al., 2013). To analyze the variation between samples, beta diversity measurements can be applied. To understand differences between microbial communities, both taxonomic and non-taxonomic approaches can be used. Ensuring reliable comparisons between the structure of different microbial assemblages based on taxonomy requires high-quality taxonomic data to be obtained from databases (based on sequences from marker genes, e.g. the 16S rRNA gene); therefore, taxonomy-based similarity measures such as UniFrac, which calculate the phylogenetic distance between community members (Lozupone & Knight, 2005), may be less useful in samples where much of the diversity is poorly characterized and hence the phylogenetic relationships are uncertain. Alternatively, traditional ecological beta diversity measures which ignore taxonomic relatedness between species, such as the Bray-Curtis similarity index, can be employed (Kuczynski et al., 2010). There is some rationale to such an approach, as due to evolutionary processes such as HGT, phylogenetic relatedness of microbial taxa does not guarantee similar ecological function.

1.5 Diversity-Function Relationships and the Importance of Ecological Theory

Simply characterizing the within-sample or between-sample diversity of microbial ecosystems is, however, insufficient to understand their important functional characteristics. Often studies will state diversity measures, α or β, as an endpoint in ecological studies but, without sufficient context, the diversity measures prove little as stand-alone measurements. Shade (2017) argues that simply stating the diversity metric of a given site provides little information about the functioning of the whole ecosystem, or about key properties such as stability or resilience,

productivity or susceptibility to invasion. It is clear that sample composition and the variation between samples do not map directly to the function of a community and do not determine microbial ecosystem success. To determine, and eventually understand mechanistically, the relationships between such properties and the richness, species distribution and taxonomic properties of these ecosystems, the application of ecological theories developed originally by macroecologists can be productive (Prosser et al., 2007). Macroecological theories which have been tested in a microbial context include the species-area relationship, the productivity-diversity relationship, and the concepts of resistance, resilience and functional redundancy, while microorganism-specific theories such as the "everything is everywhere" hypothesis are also relevant (Box 1). By guiding studies of and experimentation with microbial ecosystems with such theoretical approaches, the full power of today's molecular approaches can be exploited and true understanding and predictive power may eventually be attained (Widder et al., 2016).

Microbial communities play fundamental roles in nature through their contribution to ecosystem services, and the functional diversity of the 10^{30} microorganisms in the biosphere (Whitman, Coleman, & Wiebe, 1998) helps make Earth habitable for "higher" organisms. But, how do we link the function of a system to the known and unknown microbial species in a particular environment? Microbial ecologists are currently exploring ways in which to develop integrated approaches in order to link the structure of microbial communities to overall function of the ecosystem (Graham et al., 2016). Understanding the relationship between function of a system and the microbial species present (and how this changes over time) can be challenging; however, one way in which to explore how the microbial diversity drives certain processes is to examine well-understood systems with predictable functions and attribute the performance/function to the active microbial consortia. Here we will examine well-understood systems carrying out anoxic degradation (anaerobic digesters), nutrient cycling (sediment-water microcosms) and co-digestion of nutritional input (the human gastrointestinal tract) and assess the function (and therefore importance) of key microbial players within the diverse communities known to inhabit them.

2. ATTRIBUTING FUNCTION TO MICROBIAL DIVERSITY IN WELL-UNDERSTOOD SYSTEMS I - ANAEROBIC DIGESTION

2.1 Microbial Ecology of Anaerobic Digestion Systems

Anaerobic digestion (AD) is an economically viable and environmentally sustainable means of converting waste to value added products (biogas and digestate). AD is routinely employed in wastewater treatment where sludge resulting from previous aerobic treatment stages is digested (in the absence of oxygen) by microorganisms which convert large, polymeric substances to methane (CH_4) and carbon dioxide (CO_2). The interactions between bacterial and archaeal community members in AD form an ideal study system to analyze the structure and function of complex communities in a controlled manner (Peura et al., 2015). AD is a robust biotechnology that has been used to treat wastes from a range of sectors including domestic and industrial wastewaters, paper and pulp processing, food waste as well as agricultural residues and animal wastes (Kamali et al., 2016). Recently, various bioreactor designs have been developed to enhance the efficiency of AD, as well as utilizing co-digestion of combined wastes (Gagliano et al., 2015). However, biogas from AD has been recorded as first being used industrially in the late 19th Century, illustrating that many current microbial biotechnologies are based upon long-standing, if poorly-understood, approaches (McCarty, 1981).

Anaerobic digestion and carbon breakdown in natural anaerobic environments where methane generation is common (freshwater and marine sediments, glaciers and peatbogs) occurs in a stepwise process (Bräuer et al., 2006; Anesio et al., 2017). As the stages of the breakdown process are known, specific community members can often be allocated to specific functions within the system. During the degradation of carbon in anoxic conditions, facultative or strictly anaerobic *Bacteria* mediate the first three main reactions: hydrolysis, acidogenesis and acetogenesis (see Fig. 3). Following acetogenesis, the methanogenic *Archaea* (methanogens) facilitate the production of methane from the end products of bacterial metabolism; this results in a CO_2/CH_4 mixture termed biogas. In a closed system, such as a methanogenic bioreactor, free CO_2 can be reabsorbed and used in further hydrogenotrophic methanogenesis to form methane.

During hydrolysis, organic matter is degraded from larger polymeric material into component molecules by hydrolytic members of the anaerobic community (Fig. 3). Long chain polymers cannot be transported across the bacterial cell membrane, therefore members of the hydrolytic community export extracellular enzymes into the environment to break down bio-available

polymers (Weimer, Russell, & Muck, 2009). The hydrolytic phase is often rate limiting and places microbial growth kinetics under stress; however, microorganisms capable of performing hydrolysis are often resistant to challenging abiotic factors (Venkiteshwaran et al., 2015; Adekunle & Okolie, 2015). The rate of hydrolysis is dependent on numerous factors, including rate of extracellular enzyme production, substrate particle size and the rate of diffusion of molecules with the reactor. The microbial species able to metabolise the large, organic polymers are phylogenetically diverse and include *Bacteria* from across taxonomic groups. Many hydrolytic organisms are found within the phylum *Firmicutes* and include *Clostridium, Streptococcus* and *Bacillus* species. Following depletion of freely available carbohydrates, amino acids (from protein lysis) are utilized as energy sources by hydrolytic *Bacteria* (Lubken et al., 2007). Importantly, the taxonomy of the community can differ between reactors or over time based on local or temporal biogeography, as well as due to operational or environmental factors. Providing the function of the microbial biomass within the system can still be elucidated, we therefore have a means of linking taxonomy to function and assessing the potential for functional redundancy (Box 1). Additionally, the use of different feedstocks (such as industrial or agricultural wastes) or operational parameters in AD will alter the microbial community structure, and in this case taxonomic changes can be mapped to changed functional properties (Witarsa et al., 2016).

Following hydrolysis of organic material, monomers are rapidly converted to short chain volatile fatty acids (SCVFAs) by acidogenic bacteria (Fig. 3). Routinely, acetic acid (CH_3COOH), propionic acid (CH_3CH_2COOH) and butyric acid [$CH_3(CH_2)_2COOH$] are produced by organisms such as *Clostridium, Flavobacterium, Butyricicoccus* and *Anaerobsolibacter* (Wang et al., 2014; Gao et al., 2016). Additionally, some intermediate alcohols and organic acids can be produced during acidogenesis. Acidogenesis is generally a rapid phase of AD and can cause a decrease in reactor pH if fatty acids accumulate. Organic overload, the introduction of toxic metals and temperature change can all stall reactors following acidogenesis. Following acid production, volatile fatty acids/organic acids are converted into acetate by acetogenic *Bacteria* via reduction of carbon dioxide (CO_2) with an electron source, [predominantly an organic acid, although hydrogen (H_2) can also be utilized] (see acetogenesis in Fig. 3). Both H_2 and CO_2 can accumulate as intermediates during acetogenesis through the action of fermentative *Bacteria*. The end-products of the bacterially-mediated degradation phases are then bioavailable as substrates for methanogenesis. During methanogenesis, the key functional prokaryotic group involved in anaerobic carbon catabolism

switches from the *Bacteria* to the *Archaea* as the methanogens reduce one-carbon compounds produced in previous reactions to methane (CH_4).

Methanogens have evolved to thrive in a particularly narrow environmental niche, and as a result of this, have a limited substrate spectrum for their metabolism. Taxonomically, the majority of methanogens, including those in the family *Methanobacteriaceae*, are hydrogenotrophic, reducing CO_2 with H_2 to yield methane and water. The anaerobic biodegradation of organic matter leads to free H_2 which, if allowed to accumulate, can inhibit anaerobic breakdown by forcing an unfavourable thermodynamic equilibrium between organisms. Methanogenesis can be slowed or cease completely if free hydrogen levels rise beyond 10^{-4} atm (Venkiteshwaran et al., 2015). Importantly, in a natural system, the electron acceptor cascade readily changes with sediment/digestate depth, resulting in methanogenesis within the deepest layers, following exhaustion of other oxidants (Adhikari et al., 2016). Both acetogens and hydrogenotrophic methanogens act as hydrogen-sinks, thus preventing a build-up of hydrogen and helping to maintain optimal ecosystem functioning through preventing ineffective nutrient cycling and organic degradation. However, a competitive relationship forms between the acetogens using the reductive H_2/CO_2 pathway (Fig. 4, reaction A) and hydrogenotrophic methanogens (Fig. 4, reaction C), which are both competing for H_2 to yield either acetate or methane. Methanogens can tolerate a lower critical threshold of hydrogen than acetogens; this, coupled with the fact that the reduction of CO_2 with H_2 oxidation provides a greater energy yield for methane than it does acetate, means that hydrogenotrophic methanogens often act as the primary hydrogen-utilising organisms in anaerobic sediments and soils (Ragsdale & Pierce, 2008). Due to this competitive relationship, acetogens (which are metabolically versatile) often resort to utilizing alternative pathways, whereby they can produce acetate from fermentative by-products such as sugars, aldehydes, aromatic compounds and alcohols (Ragsdale & Pierce, 2008). Conversion of larger molecules (e.g fatty acids) to acetate results in energetically unfavourable reactions, which are made favourable through coupling to methanogenesis (Fig. 4, reaction B).

Conversely, a syntrophic relationship occurs between acetoclastic methanogens (Fig. 4, reaction D) and the acetogenic *Bacteria*. Acetoclastic methanogens utilise the acetate produced by acetogens to produce methane and CO_2, while the acetogens can then utilise the CO_2 produced during methanogenesis as a precursor for further acetate synthesis. Although more limited in its taxonomic distribution, acetoclastic methanogenesis is thought to be of greater

importance in natural and host-associated ecosystems, being responsible for up to two-thirds of annual biogenic methane production (Fournier & Gogarten, 2008). Moreover, acetoclastic methanogens seem to be predominantly responsible for methane production from well-oxygenated soils, where they most likely inhabit anoxic microenvironments rather than exhibiting resistance to oxygen (Angle et al., 2017). The relatively recent (last *ca*. 500 million years) acquisition by the ancient methanogen lineage of genes necessary for acetoclastic methanogenesis - probably from a cellulolytic organism (Fournier & Gogarten, 2008) - is likely to have had a major effect on global methane biogeochemistry. Acetoclastic methanogenesis is also an important pathway in reactor-based anaerobic digestion, although seems to be more sensitive to inhibitors such as free ammonia than the hydrogenotrophic pathway (De Vrieze et al., 2016).

It should be noted that there can be additional methanogenic pathways occurring in sediments and other anaerobic zones. Methylotrophic methanogens, those which utilise methanol and other methylated compounds, such as methanethiol (CH_4S) and methylamine (CH_5N), can encode specific enzymes to convert such compounds to methane. Recently, Mayumi *et al.* (Mayumi et al., 2016) predicted a fourth mechanism of energy generation by methanogens, termed "methyoxydotrophic" methanogenesis. This mode of methanogenesis is novel as it allows the degradation of complex methoxylated aromatic compounds (MACs) by methanogens (particularly *Methermicoccus* species) and couples this reaction to CO_2 reduction, demethylation of methyl ethers (*O*-demethylation) and potentially central metabolic pathways involving acetyl-coenzyme A (ACoA). It is clear from such recent studies that our knowledge in regard to methanogenic biochemical pathways remains incomplete.

During the anaerobic degradation of organic compounds, conductive surfaces can facilitate Direct Interspecies Electron Transfer (DIET) between degradative organisms and species which carry out the terminal electron-accepting processes, such as methanogens (Lovley, 2017). In methanogenic soils, *Geobacter* species which can transfer electrons by DIET are highly metabolically active, as are *Methanothrix* species which can reduce CO_2 to methane using DIET-derived electrons (Holmes et al., 2017). DIET also appears to be an important means of transferring electrons to methanogens in some anaerobic digesters (Morita et al., 2011), and addition of conductive materials to such reactors can increase methanogenesis potential (Baek et al., 2015). It also appears that facilitation of DIET in AD systems can help overcome the inhibition due to acidification and increase in the partial pressure of H_2 (Zhao et

al., 2017), or due to the presence of electron acceptors such as sulphate which normally yield favourable thermodynamics compared to methanogenesis (Li et al., 2017). DIET is therefore an important, though still incompletely understood, ecological process in both anaerobic soils and sediments and AD systems.

Despite our still-incomplete knowledge of the biochemical pathways in AD and their taxonomic distribution, the controllability, replicability and ease of sampling of AD systems make them an enticing target for the exploration microbial community structure-function relationships and dynamics. Through analysis of AD-derived data, insights can be gained into the role of each metabolically active microbial group (e.g. hydrolysers, acetogens, methanogens), and hypotheses linking function to taxonomic diversity can be developed. The challenge is then to test these hypotheses in controlled and manageable microbially-driven biotechnological systems, with the eventual aim of functional control through the understanding and manipulation of microbial ecology (Narayanasamy et al., 2015).

2.2 Monitoring Anaerobic Digestion Through –omics Technologies

The application of -omics-based technologies to monitor the AD process has helped to further the understanding of how AD systems operate in regard to microbial community structure and function. Metataxonomic studies have shown that the microbial composition of inocula, the effects of pre-treatment, and operating conditions of the bioreactor can all influence the structure of microbial communities present and influence performance (Vanwonterghem et al., 2014). Utilization of the metataxonomic data allows insights into how to stabilise AD performance, and may lead to novel isolation strategies to produce tailored "starter communities" to bioaugment reactors and enhance a particular function (e.g. an optimized hydrolytic community, as hydrolysis is often rate limiting). Additionally, with improvements in NGS technologies, longer sequence reads may be able to provide greater taxonomic data and functional characterization at deeper levels.

The use of metatranscriptomics in AD has allowed the study of *in situ* gene expression of the microbial community during degradation of wastes, including determining the impacts of disturbance within the reactor on the microorganisms' gene expression. Stressful operating parameters, such as volatile fatty acid (VFA) accumulation, leading to a decrease in pH can be monitored through changes in mRNA expression. Furthermore, due to the use of whole, undefined communities in AD, many of the metabolic pathways used by the microbial

consortia remain incomplete. It is thought that *Clostridium* species hold great potential in expression of enzymes used in the degradation of recalcitrant wastes and could be a target to improve yield of monomers for further AD reactions (Guedon, Desvaux, & Petitdemange, 2002). Transcripts involved in enzyme synthesis for use in acid production and conversion to acetate by fermentative bacterial species have also been monitored in AD systems, along with archaeal mRNA levels involved in the generation of methane (Zakrzewski et al., 2012). Despite advances in metatranscriptomic analysis and the use of mRNA expression databases, mRNA recovery from environmental samples can be low and enrichment of recovered molecules can prove challenging. However, assessment of the total gene expression proves beneficial in understanding how microorganisms respond to fluctuations in reactor conditions which, combined with metataxonomic data, allows a more systematic approach to stabilizing the community and enhancing reactor performance (Vanwonterghem et al., 2014; Williams et al., 2013). The use of metatranscriptomics to elucidate processes such as DIET in natural methanogenic ecosystems (Holmes et al., 2017) also illustrates its potential to enhance our understanding of the ecological interactions during anaerobic degradation. The applicability of metatranscriptomics to AD systems will likely become enhanced as technical developments improve the yield and quality of RNA extraction.

The use of metaproteomics techniques in methanogenic bioreactors allows characterization of the total protein complement expressed by the members of the microbial community. Although similar to assessing the metatranscriptome (mRNA levels), study of the proteome allows a powerful approach to assess metabolic pathways and regulation of AD reactions. The role of microorganisms which produce critical enzymes can be explored through proteomics, as well as identification of potentially novel enzymes expressed during digestion of wastes. A key study by Abram et al. (2011) examined the proteome of a microbial community within a digester treating industrial wastewater. The majority of proteins expressed showed predicted functions in methanogenic pathways (both acetoclastic and hydrogenotropic), glycolysis and the pentose-phosphate pathway. In conjunction with both metagenomics and metatranscriptomics, the utilization of proteome-analysis based techniques may improve AD productivity by allowing identification of critical pathways and biomarker molecules which may be expressed in response to unfavourable reactor conditions (Hanreich et al., 2013; Vanwonterghem et al., 2014). Monitoring changes in the proteome may allow AD operators to adjust reactor conditions prior to digester failure or souring (Chen, Cheng, & Creamer, 2008).

2.3 Does Taxonomic Diversity Drive Resilience and Productivity in Anaerobic Digesters?

It is thought that maintaining a high level of biodiversity within an ecosystem (both natural or engineered) may be key to promoting stability, productivity/yield and function of the system (Beyter et al., 2016); (Box 1). High species richness (number of species), along with high evenness (of relative abundance of species compared to one another) within the community can enhance functional redundancy. The resultant sharing of metabolic roles can aid in recovery of digesters following disturbances. Werner et al. (2011) analysed the relationship between bacterial community structure and bioreactor performance and found that bioenergy systems contain communities that are unique to any given reactor, but share high levels of overall stability. This work argued that the community's ability to withstand change (the level of resilience) was high amongst syntrophic organisms that promote digester function. Further to this, shifts amongst functionally redundant organisms allowed function to be maintained. Therefore, AD systems with a more even distribution of microbial species showed higher methanogenic activity and more efficient removal of organic input than systems where the species distribution was more uneven and functional redundancy was therefore reduced (Werner et al., 2011).

Additionally, Carballa et al. (2011) found that a more even and taxonomically diverse community was representative of a more optimally functioning lab-scale anaerobic digester, with high bacterial evenness correlating to higher methane yields under mesophilic conditions. Their work also showed that the populations of both *Bacteria* and *Archaea* in the reactors were highly dynamic, even when methanogenic performance was stable. Similarly, the work of Fernández et al. (1999) showed that a highly dynamic microbial community composition could be compatible with overall system performance in a methanogenic bioreactor. Subsequent work by the same authors (Fernández et al., 2000; Hashsham et al., 2000) showed that more taxonomically-stable communities were less resilient in their functional response to a glucose-loading perturbation compared to those which exhibited taxonomic variability. It appears that the methanogenic consortia are particularly sensitive to changes in environmental conditions (Turker et al., 2016), so maintaining optimal reactor conditions to prevent disassembly or failure of this sub-community may be critical to ensure reliable biogas generation. Further work into community structure-function relationships will allow communities to be optimized and promote enhanced and beneficial functions in AD.

3. ATTRIBUTING FUNCTION TO MICROBIAL DIVERSITY IN WELL-UNDERSTOOD SYSTEMS II - NUTRIENT-CYCLING MICROBIAL MICROCOSMS

While AD systems are important biotechnological tools which can also be used at the lab scale for replicated manipulation experiments to test fundamental features of their microbial ecology, they are essentially linear (input-output) systems which at small scale are most easily operated as a batch process. Moreover, they contain only a subset of microbial functional diversity: those species involved in anaerobic, non-photosynthetic degradation processes. At the global scale, microorganisms are involved in the cycling of all the nutrients essential for life, and these processes are ultimately driven by the energy derived from photosynthesis (Falkowski, Fenchel, & Delong, 2008). Therefore, a more complete picture of microbial diversity-function relationships could potentially be gained by studying analogous nutrient-cycling ecosystems, in which photosynthetic processes play an important role, at the lab scale (Free & Barton, 2007). Such a model system is provided by a long-established laboratory microcosm system, the Winogradsky column.

3.1 Using Nutrient-Cycling Microcosms to Study Structure-Function Variability in Complex Microbial Communities

Microcosms are a unique tool for microbial ecologists to test ecological theory (Jessup et al., 2004; Prosser et al., 2007; Widder et al., 2016). Microcosms can develop rapidly, be replicated many tens to hundreds of times, and are easy to manipulate or control, while preserving ecological interactions that are observed in the field. This allows microbial ecologists to perform experiments that are either not possible, or extremely difficult to do for macro-ecologists. For example, microcosms can be used to monitor the evolution of microbial lines over many generations in a relatively short timeframe, or can be used to investigate the effects of perturbation on communities through changes in environmental conditions (such as the addition of a pollutant). Their use is also a method of moving microbial ecology forward from the “stamp-collecting” approach of high-throughput characterization of natural samples to the more focused targeting of generalized understanding of diversity-function relationships (Prosser, 2012; Widder et al., 2016).

The classic Winogradsky column microcosm (Fig. 5), invented in the 1880s by Sergei Winogradsky, was originally intended to enrich for anaerobes (Madigan et al., 2011; Dworkin, 2012). It is a water-sediment microcosm, made with natural materials sampled from aquatic environments, and thereby containing all the microbial diversity required for a functioning, nutrient-cycling ecosystem. The column is supplemented with a carbon source (cellulose) to drive the development of anaerobic conditions via organic degradation, and a sulphur source (typically $CaSO_4$) which acts as the primary terminal electron acceptor in the system, and enhances the anaerobicity of the sediment layers in which H_2S is produced by sulphate reduction. After exposure to light and as the added organic matter is degraded, the microbial communities self-organise vertically into distinct layers, with aerobic conditions in the air-water layer and anaerobic conditions in the deeper sediment layer. This vertical redox gradient is both a consequence of the microbial activities and determines the functions which can proliferate in the different layers, thereby exemplifying the biological-environmental feedback which is an essential property of natural microbial ecosystems (Free & Barton, 2007). The organic degradation pathways in this initial stage of the system are analogous to those found in AD (Section 2), although, due to its thermodynamic favourability, sulphate reduction predominates over methanogenesis as the terminal electron-accepting process (Muyzer & Stams, 2008). Subsequently, once the initial organic degradation phase is complete, nutrient cycles driven ultimately by energy derived from photosynthesis emerge (Fig. 5). This is a closed nutrient-cycling system, and so is not affected by species immigration or emigration. Apart from that, the Winogradsky column is the closest approximation to a real aquatic ecosystem that can be used in the lab.

The microbial diversity in the Winogradsky column is driven by the provision of light nutrients, which select organisms involved in photosynthesis and the carbon and sulphur cycles, respectively (Fig. 5). Hence, the functions of the most abundant microorganisms present in the resulting communities can be assumed to be involved in these core pathways. The upper aerobic layers contain oxygenic phototrophs involved in primary production, such as cyanobacteria and eukaryotic algae. As the concentration of oxygen decreases vertically down the column, facultative anaerobes and phototrophic Sulphur Oxidizing Bacteria (SOB) proliferate. The SOB are further stratified by oxygen concentrations such that nonsulphur phototrophic bacteria (e.g. *Rhodospirillales*) proliferate in the upper layers, purple sulphur bacteria (e.g. *Chromatiales*) proliferate in the middle layers, and green sulphur bacteria (e.g.

Chlorobiaceae) proliferate in the lower layers (Madigan et al., 2011; Rundell et al., 2014). The lowest sediment layers contain anaerobic heterotrophic digesters and fermenters that degrade organic matter produced in the upper layers. They also contain methanogens (e.g. *Methanothrix* sp., formerly *Methanosaeta*) converting organic acids to methane, in competition for electron flux with Sulphate-Reducing Bacteria (SRB). The proliferation of SRB in the deeper sediments completes the sulphur cycle. For long-term function of the Winogradsky column, other functional groups involved in minor metabolisms also proliferate, allowing full recycling of all biologically-essential nutrients. However, due to the abundance of functional groups involved in the carbon and sulphur cycles, Winogradsky columns continue to be used as a tool for the enrichment of those microorganisms (Loss et al., 2013; Charlton, McGrath, & Harfoot, 1997).

3.2 Stochastic Community Variation Following Selective Bottlenecks

The Winogradsky column model has been used to look at microbial community assembly, the effects on diversity that this produces, and the effects this has on ecosystem function (Pagaling et al., 2014; Pagaling et al., 2017). These studies were aimed at testing a variety of ecological theories in a complex microbial system, including the resilience of community structure and function to initial conditions, the redundancy of different microbial species in this system, the "everything is everywhere" hypothesis and the species-area relationship (Box 1).

Replicate microcosms (n=100) were set up identically to test whether trajectories of microbial assembly were deterministic or sensitive to stochastic fluctuation (Fig. 6). Initially, a large drop in diversity (compared to the original environmental inoculum) is observed, which is associated with the transfer of environmental species to a lab environment, where exposure to continuous light, high levels of organic input (cellulose) and sulphate, which is generally low in freshwater ecosystems, are imposed. A few species are selected due to their ability to proliferate in these conditions, with members of the *Firmicutes*, which are associated with the degradation of microcrystalline cellulose, dominating the heterotrophic degrader communities. The minimum diversity state of the community is concurrent with the maximum production of sulphide (Fig. 6), which will also have a strong selective effect as not all species can tolerate such conditions, and seems to be largely deterministic in terms of community composition (Pagaling et al., 2017). Following this diversity minimum, diversity increases again as the communities re-organise themselves into functional groups as described above, and the system recovers to a

stable state. In the multiple replicate microcosms, the diversity of the heterotrophic degraders in the mature columns appears to split into two groups such that the microcosms produce two alternative stable states. Communities are either dominated by *Firmicutes*, or by *Bacteroidetes*. In addition, *Anaerolineae* are co-selected with the *Firmicutes*, while *Treponema* (phylum *Spirochaetes*) and *Verrumicrobia* are co-selected with the *Bacteroidetes*. This suggests the presence of functionally-redundant microbial community compositions, both of which are compatible with a stable, nutrient-cycling system. Moreover, a microcosm size effect is evident, with smaller microcosms producing communities dominated either by *Firmicutes* or *Bacteroidetes*, and larger columns consistently producing *Firmicutes*-dominated communities (Pagaling et al., 2017). However, a classic species-area relationship between microcosm size and overall community richness (Box 1) is not observed, possibly because of the earlier diversity bottleneck caused by the selective effects of cellulose addition and sulphide production.

Interestingly, the alternative community compositions of heterotrophic degraders in the final microcosm states has downstream consequences for the diversity of SRB, with elevated levels of *Sulfurospirillium* in the *Firmicutes*-dominated communities, while *Desulfovibrio*, *Desulfobulbus*, *Desulfobulbaceae*, *Desulfobacteraceae* and *Desulfarculaceae* are elevated in *Bacteroidetes*-dominated communities. This may be due to differences in organic acid production by the different heterotrophic degrader communities, which affects substrates available for sulphate reduction (Leschine, 1995; Muyzer & Stams, 2008).

3.3 Source Community Variation is Secondary to Divergence Following Selection

From the aformentioned studies, it is apparent that there is strong selection of particular species from the original environmental inoculum, leading to the diversity patterns seen in mature microcosms. This system could therefore be used to test the Baas-Becking hypothesis that "everything is everywhere; the environment selects" (Martiny et al., 2006). Under this hypothesis, transfer of the total microbial diversity (the inoculum) into the same environment (the microcosm) would be expected to yield taxonomically-similar mature microcosm communities, as the same specific functions would be repeatedly selected. Alternatively, if sufficient redundancy is present within those functions required during microcosm development, microbial taxonomy may differ across the mature microcosms as long as all

functional groups are present. In an experiment to test this directly, triplicate sterile (autoclaved) microcosms were inoculated with environmental source communities taken from different freshwater aquatic environments around Scotland, and the resulting microcosm community similarities were quantified (Pagaling et al., 2014). In this experiment, the community structure in the mature microcosms is highly variable, such that the final community compositions become more different from each other than the environmental inocula are, despite selection in the common microcosm environment (Fig. 7). A signal of the community's previous history, i.e. where the source inoculum has come from, is retained, and determines the direction of divergence to develop the distinct final microbial communities. Variability within the triplicate microcosms inoculated with the same source sediment is also observed, consistent with the highly-replicated experiment from a single inoculum. This may be due to the fact that dominant members of the microbial communities in mature microcosms were originally rare members in the inoculum, as dilution of the inoculum leads to greater inter-replicate variability between microcosms, especially for the numerically-rarer archaeal population (Pagaling et al., 2014).

These experiments suggest that strong selective bottlenecks can cause divergence in subsequent trajectories of community assembly once the cause of the bottleneck is removed. This allows proliferation and re-diversification of other functional groups, in this case heterotrophic degraders not specialized in cellulose degradation and other functionalities required for long-term nutrient cycling. Stochastic variation in the populations of these microorganisms present in the bottleneck community between individual microcosms results in the variation in community structure seen in the mature microcosms. Moreover, non-linear amplification through community and environmental feedbacks, evolutionary history, mutation, low-frequency exit from the non-growing state, birth-death dynamics and viral predation (Bohannan & Lenski, 2000; Buerger et al., 2012) could contribute to the differences in the mature microcosm communities. As stable, active microcosms seem to result in all cases, it could be argued that this is strong evidence of functional redundancy across the range of functional groups essential for the system. A disadvantage of the complexity of this microcosm system is that a simple measure of ecosystem function is difficult to find; however, the redox potential gradient generated by the sum of the microbial activities in the system can be measured as a proxy. When this was measured in the sterile microcosms inoculated with different source communities, redox potential was seen to vary widely with inoculum, and to

some extent between replicates derived from the same inoculum (Pagaling et al., 2014). Therefore, in this system at least, variation at the taxon level can have a substantial effect on a measure of community function, possibly as a result of the complex interactions between different functional groups which contribute to the redox potential gradient. This is in contrast to the clear functional redundancy observed in simpler systems such as AD (Fernández et al., 1999), indicating that the mapping between microbial taxonomy and the resulting functionality may be more or less clear depending on the complexity of the study system.

4. ATTRIBUTING FUNCTION TO MICROBIAL DIVERSITY IN WELL-UNDERSTOOD SYSTEMS II - MICROBIOTA OF THE MAMMALIAN GI TRACT

Compared to environmental microbiota or those organisms found in industrial biotechnology systems such as AD reactors, the microbial species which inhabit the mammalian gut are relatively well characterised in terms of cultured representatives and detailed functional studies. This is largely due to the fact that these organisms, particularly those with the potential to cause disease, have long been a subject of intensive investigation, and many of our laboratory culturing techniques have been developed to isolate and culture them. However, the advent of culture-independent methods to study their communities has facilitated a much greater understanding of the total diversity of these systems, as well as an appreciation of the benefits the majority of the GI tract microbiota bring to the host.

4.1 Gut Microbiota and the Holobiont Concept

In the last 10 years, there has been an explosion of interest in the microbiota associated with mammalian hosts, especially humans. This is true in both the scientific literature, stimulated by research efforts such as the Human Microbiome Project (Human Microbiome Project Consortium, 2012; Turnbaugh et al., 2007), and in the public consciousness via popular science literature (Yong, 2016). The concept of the microbiota as a constituent part of the human body, first proposed by Lederberg (Lederberg, 2000), has been taken forward through the widespread application of metataxonomic, metagenomic, metaproteomic and (meta)metabolomic techniques to large cohorts of individuals from human populations (Yatsunenko et al., 2012; Turnbaugh et al., 2009a) and manipulated laboratory animals (Turnbaugh et al., 2009b), and smaller studies of wild populations of mammals ranging from mice (Maurice et al., 2015) and squirrels (Ren et al., 2017) to giant pandas (Xue et al., 2015). The greatest focus has been on

the microbiota of the gastro-intestinal (GI) tract (Glendinning & Free, 2014), the individuals of which, concentrated primarily in the colon, outnumber those at other body sites by at least an order of magnitude (Sender, Fuchs, & Milo, 2016). Although the work of Sender and colleagues suggests that the oft-quoted 10:1 ratio of microbial to host cells in the human body is actually closer to 1:1 in reality, this diverse population of microorganisms nevertheless contributes a vast amount of additional metabolic and genetic potential to the combined host-microbial "hologenome" (Zilber-Rosenberg & Rosenberg, 2008).

The mature human GI tract microbiota is dominated by the Phyla *Bacteroidetes* and *Firmicutes*, with smaller contributions from the *Proteobacteria*, *Actinobacteria* and *Verrucomicrobia* (Shin, Whon, & Bae, 2015). These taxonomic distributions establish themselves over the course of 1-2 years following birth, with a series of dynamic changes occurring as the infant develops and is weaned (Sekirov et al., 2010). Using gnotobiotic ("germ-free") mice, it has been possible to show via metabolomics that a wide range of mammalian blood metabolites is affected by the gut microbiota, with a large number present only when a normal gut microbial community is established (Wikoff et al., 2009). One of the most well-known and important physiological functions of the GI tract microorganisms is the fermentation of complex carbohydrates to short chain fatty acids (SCFAs), but the microbiota is also responsible for metabolism of amino acids, lipids, and phytochemicals such as polyphenols (Krishnan, Alden, & Lee, 2015). Metagenomic analyses of human faecal samples have been instrumental in identifying the pathways involved in these metabolic transformations, and their co-occurrence in individual hosts, leading to the potential for cross-feeding (Vital, Howe, & Tiedje, 2014). As well as contributing directly to host nutrition, such activities can also influence the development and function of distal body sites, for instance the production of immunomodulatory metabolites from aromatic amino acids, which can also affect communication between the gut and the nervous system (Vital, Howe, & Tiedje, 2014). Such mechanisms may be involved in the numerous interactions observed between GI tract microbiota composition and various neurological disorders via the "gut-brain axis" (Collins, Surette, & Bercik, 2012).

Although the explosion of knowledge of, and interest in, the microbiota of the GI tract has been driven by the emergence of high-throughput culture-independent methods, there remains a need for culture-based studies to demonstrate causal relationships between members of the microbiota and host phenotypes, and to elucidate the relevant molecular or physiological

interactions. To this end, novel culturing techniques can play a vital role (Sommer, 2015). Such methods include the use of devices such as diffusion chambers and iChips to allow the cultured microorganisms to access essential compounds present in their preferred niche (Kaeberlein, Lewis, & Epstein, 2002; Nichols et al., 2010), the development of novel and niche-specific media (Goodman et al., 2011; Lagier et al., 2012) and selection for diverse spore-forming genera using ethanol (Browne et al., 2016). In all of these approaches, culture-independent methods can also play a role by allowing choice of a suitable selection medium based on taxonomy or facilitating in-depth genomic characterisation. It is also noteworthy that there is a significant proportion of culturable GI tract microbiota which are under-represented in culture-independent studies (Rettedal, Gumpert, & Sommer, 2014): the formation of difficult-to-lyse spores or the presence of resistant cell membranes are likely to contribute to the poor representation of these species in studies which rely on generalised DNA extraction methods (Sommer, 2015).

It is important to remember that *Bacteria* and *Archaea* are not the sole constituents of the gut microbiota. The complex ecosystem that is the GI tract microbiome also includes microeukaryotes and viruses, the latter targeting both the host (viruses) and the microorganisms (bacteriophages and archaeophages) (Scarpellini et al., 2015). Interestingly, in contrast to the best-studied environmental ecosystems (Thingstad, 2000), bacteriophages in the gut seem to be relatively less diverse and exhibit greater temporal stability and a generally lysogenic (temperate) lifestyle (Reyes et al., 2010; Reyes et al., 2012). Alongside the host-targeting viruses can exist various other gut pathogens such as eukaryotic intestinal parasites (e.g. helminths) and pathogenic bacteria. All of these deleterious microorganisms can interact with, perturb and be affected by the commensal microbiota and the host immune system (Glendinning et al., 2014; Bancroft, Hayes, & Grencis, 2012; Zaiss & Harris, 2016), and the resistance to colonisation by, and control of, pathogens and pathobionts is an additional major function of the gut microbial community (Buffie & Pamer, 2013; Kamada et al., 2013). Thus, considering the GI tract and, by extension, the entire human body as a "superorganism" composed of the cells, genomes and activities of all its constituent microorganisms, viruses and parasites, interacting via multiple pathways and influencing each other's evolution (Salvucci, 2012), yields our updated, much more detailed but still poorly-understood concept of the holobiont (Zilber-Rosenberg & Rosenberg, 2008).

4.2 Dysbiosis

Although the GI tract microbiota is generally stable over long periods of time in healthy adults (Faith et al., 2013), perturbations of its composition can occur due to various endogenous and exogenous factors, which result in a state termed "dysbiosis" (Tamboli et al., 2004). Dysbiosis can be considered as a shift in the natural balance between well-tolerated, commensal or beneficial microorganisms and those species which can be deleterious to the host (Walker & Lawley, 2013). This disruption can be due to host-specific factors such as host genetics, immune deficiency (Maharshak et al., 2013) and colorectal cancer (Marchesi et al., 2011), or to external influences such as antibiotic usage (Buffie et al., 2012; Lawley et al., 2012) and diet (Carmody et al., 2015). Although these different influences on microbiota composition can result in equally different dysbiotic states, a reduction in overall microbial diversity and an elevation in abundance of members of the Phylum *Proteobacteria* constitute two common signatures of dysbiosis (Walker & Lawley, 2013; Shin, Whon, & Bae, 2015).

The mechanism and consequences of the onset of dysbiosis are likely to vary depending on the causative agent. The most clear-cut pathway is the administration of broad-spectrum antibiotics, in particular those such as clindamycin which can target the core anaerobic species of the GI tract. Administration of clindamycin to leads to a rapid (within 2 days) and almost complete eradication of the core *Firmicutes* and *Bacteroidetes* species of the mouse caecum, a dramatic reduction in Shannon diversity (from 4-6 to <1) and strong selection of a handful of genera such as *Akkermansia*, *Blautia* and members of the *Enterobacteriaceae* (Buffie et al., 2012). This impoverished and disrupted community state has lost the colonisation resistance normally imparted by the microbiota, making clindamycin-treated mice, unlike their untreated counterparts, highly sensitive to challenge with *Clostridium difficile* over sustained periods (Buffie et al., 2012). Subsequent attempts to clear *C. difficile* infection (CDI) with additional antibiotic treatment have high relapse rates due to the concurrent collateral damage to members of the normal GI tract microbiota which are required to restore colonisation resistance, although the use of less broad-spectrum compounds such as fidaxomycin, which have fewer effects against the endogenous members of the *Firmicutes*, *Bacteroidetes* and *Actinobacteria* populations, can circumvent this problem to some extent (Louie et al., 2011; Tannock et al., 2010).

Many types of dysbiosis are associated with inflammation in the gut, and it is likely that the immune system is intimately involved in the transition to a dysbiotic state. In the healthy state, the immune system is tolerant of mutualistic and commensal microorganisms, while

discriminating against potential pathogens (Franchi et al., 2012). An inappropriate immune response could therefore be the trigger which leads to microbiota destabilisation and dysbiosis; or, alternatively, an initial microbiota disturbance via the chance presence of a particular pathogen could lead to an immune reaction and further perturbation via inflammation. Although the initial trigger may be hard to identify, this type of self-reinforcing interaction probably underlies inflammatory bowel diseases (IBDs) such as Crohn's disease and ulcerative colitis (Zhang et al., 2017a). Alleles of human genes involved in the adaptive and innate immune responses are associated with a risk of IBD (Franke et al., 2008; Hugot et al., 2001; Ogura et al., 2001), while in laboratory mice, animals lacking the Toll-like receptor (TLR)-5 or the immunoregulatory cytokine IL-10 develop spontaneous colitis (Maharshak et al., 2013; Carvalho et al., 2012). In the mouse studies, the onset of dysbiosis is associated with a reduction in microbial diversity and an increase in the abundance of members of the *Proteobacteria* (Shin, Whon, & Bae, 2015), while low-diversity states with compositions showing elevated *Enterobacteriaceae* are also observed in human IBD patients (Morgan et al., 2012; Lavelle et al., 2015). The elevation of *Proteobacteria* in such patients seems to be most marked during episodes of severe inflammation (Walujkar et al., 2014). It is possible that usage of nitrate generated by the host immune response as an electron acceptor by *Enterobacteriaceae* may allow them to outgrow fermenters such as *Bacteroidetes* and *Firmicutes* during inflammation (Winter et al., 2013), resulting in positive feedback via further inflammation (Shin, Whon, & Bae, 2015). Interestingly, both the normal and dysbiotic states of the GI tract microbiota exhibit the Lamarckian properties of evolution via the use or disuse of individual characteristics (microorganisms) and transmissibility to offspring (Rosenberg, Sharon, & Zilber-Rosenberg, 2009). These characteristics also help to make engineering of the microbiota for beneficial effects on the host a viable strategy for treating dysbiosis and other diseases which are linked to the microorganisms of the GI tract.

5. MANIPULATION OF MICROBIAL COMMUNITY DIVERSITY TO DIRECT ECOSYSTEM FUNCTION

As our understanding of the complexity of microbial communities has increased, and we have begun to appreciate how their diversity affects ecosystem function, there has been a burgeoning interest in manipulating diversity, and therefore function, for practical applications. Various levels of manipulation can be conceived. Synthetic ecology, the conceptually-simple "bottom-up" engineering of defined collections of cultured species, has been used to test ecological theory as well as for various practical applications. Single- or multi-species bioaugmentation

has been used to improve processes in wastewater treatment and bioremediation. Artificial ecosystem engineering, a "top-down" selective approach originally tested some years ago, has been suggested more recently as a method to improve host functions via manipulating the associated microbiota. Here, we review current progress with these methods and potential problems and obstacles hindering their further development.

5.1 Synthetic Ecology

In synthetic ecology, individual species known to carry out specific roles are co-cultured *in vitro* to form stable engineered microbial communities (Fredrickson, 2015). This "bottom-up" approach dictates microbial diversity to direct ecosystem function. Synthetic ecology provides a useful tool to study ecological theory (Box 1) as the synthetic communities are constructed from a few species and so are less complex than natural communities, but can still mimic interactions in nature (Shou, Ram, & Vilar, 2007), though performance may be reduced in the lab environment (Yu et al., 2016). They also have the added advantage of being easily controlled in the lab. Since synthetic ecology allows targeted design of microbial communities, it can also be used to improve biotechnological applications, and other environmentally beneficial microbial processes. It also has potential use as a medical treatment of infectious diseases, via the application of defined mixes of microbial strains for bacteriotherapy (Lawley et al., 2012).

In order to achieve stable self-sustaining, nutrient-cycling ecosystems, the functions of the individuals must be elucidated. Therefore, many of the model systems use genetically modified strains so that functions of individuals can be controlled, though some synthetic communities may be formed in combination with wild-type microorganisms. Shou et al. first developed the concept of synthetic ecology for studying cooperation by constructing a simple community of genetically engineered auxotrophic yeast to elucidate robustness and boundary parameters of that system (Shou, Ram, & Vilar, 2007; Dunham, 2007). Since then, many more synthetic communities have been used as model systems to study cooperation by observing cell-cell interactions. One study showed that defined spatial structures were required for cooperation in genetically modified *E. coli* biofilms, which led to increased persistence (increased colonization of a downstream environment) and biomass production (Brenner & Arnold, 2011). Similarly, spatial structure was required for cooperation between *Salmonella enterica*

ser. Typhimurium and an *E. coli* mutant (Harcombe, 2010). Recently, synthetic communities of genetically engineered *E. coli* populations (consisting of activator and repressor strains) were used to observe genetic and population-level oscillations when the strains were grown together (Chen et al., 2015). Genetically modified auxotrophic strains of *E. coli* were used to demonstrate that syntrophy encouraged cooperative relationships to evolve to avoid collapse of the community (Hosoda et al., 2011); that cooperative phenotypes show more growth on average (Wintermute & Silver, 2010), and that stronger cooperative relationships are formed if the nutrients that are exchanged are biosynthetically costly (Mee et al., 2014). Synthetic communities of genetically modified yeast strains were used to investigate the effects of cheaters on cooperation (Gore, Youk, & van Oudenaarden, 2009; Waite & Shou, 2012). Furthermore, the maintenance of cooperation was studied among genetically engineered *E. coli* populations of producers and non-producers of "common good" (Chuang, Rivoire, & Leibler, 2009; 2010).

In addition, synthetic communities have been constructed to investigate other microbial interactions including predator-prey systems (Balagadde et al. 2008), extinction and commensalism (Hu et al., 2010). Increasingly complex synthetic communities have been constructed, including a bacterial-eukaryote synthetic community consisting of a genetically modified *E. coli* strain and an amoeba, *Dictyostelium discoideum* (Kubo et al., 2013), and synthetic ecosystems consisting of bacteria, yeast, engineered mammalian cells and plants (Weber, Daoud-El Baba, & Fussenegger, 2007).

To our knowledge, there are few synthetic communities that exclusively use wild-type microbial isolates. The soil bacteria *Azobacter vinelandii*, *Bacillus licheniformis* and *Paenibacillus curdlanolyticus* were used to form a purely synthetic community as these bacteria do not normally interact in nature (Kim et al., 2008). Each bacterium performed a unique function that was essential for the survival of the community; *Azobacter vinelandii* fixes nitrogen, *Bacillus licheniformis* degrades penicillin G, and *Paenibacillus curdlanolyticus* provides carbon through the degradation of cellulose. Importantly, these bacteria were not able to grow as a planktonic co-culture, but the community was stabilised once a specific spatial structure was imposed using a microfluidic device which allowed intracellular metabolic communication without competition for space (Kim et al., 2008). Synthetic communities performing methane oxidation have also been established. The simple (2/3 species)

communities consisted of methanotrophs, while the complex (50 species) communities contained methanotrophs, non-methanotrophic methylotrophs and non-methylotrophic heterotrophs that were isolated from Lake Washington sediments (Yu et al., 2016). Notably, though, the more complex communities rapidly lost species when incubated, illustrating the difficulty of constructing complex, stable communities as found in natural ecosystems. A synthetic community consisting of *Pseudomonas reinekei*, *Wautersiella falsenii*, *Achromobacter spanius* and *P. veronii* – which were isolated from sediments of the river Spittelwasser, Germany – were grown in co-culture for the degradation of 4-chlorosalicyclate (Pawelczyk et al., 2008), an intermediate in the degradation of the toxic compounds chloro-dibenzofurans and chloro-dibenzo-p-dioxins (Bobadilla Fazzini et al., 2009). 4-chlorosalicyclate was only utilized by *P. reinekei*, while *A. spanius* and *P. veronii* used intermediates of 4-chlorosalicyclate degradation, and *W. falsenii* was a necrotiser living on constituents of the other community members, hence, all four members of the community were required for complete degradation of 4-chlorosalicyclate (Pawelczyk et al., 2008).

A potential application of synthetic ecology is to improve a number of biotechnological processes (Brenner, You, & Arnold, 2008; Pandhal & Noirel. 2014; Shong, Jimenez Diaz, & Collins, 2012). Much work has been done using synthetic ecology to improve biofuel production from ligno-cellulose (reviewed in (Jouzani & Taherzadeh, 2015)). Microbial consortia are required to degrade ligno-cellulosic biomass to ethanol since no single microorganism can ferment all the sugars produced from the hydrolysis of cellulose. Therefore, synthetic communities could be constructed so that each microorganism exclusively degrades one sugar, which would allow population-level coordination and control of ecosystem stability and dynamics (Shong, Jimenez Diaz, & Collins, 2012). This approach results in more efficient processing [e.g. reduction of lag time: (Xu & Tschirner, 2011], and increased community stability: (Unrean & Srienc, 2010; Eiteman, Lee, & Altman, 2008) and higher yields compared to their mono-cultured counterparts (He et al., 2011).

Production of biomass that has traditionally involved the growth of mono-cultures also has the potential to benefit from synthetic ecology. For example, the production of algal biomass for use in biofuels, dyes and food supplements has usually required the cultivation of single species. However, growth of algae is improved when co-cultured with bacteria that have complementary functional traits by increasing community stability and increasing resistance to

predators and contaminants (Kazamia et al., 2014). Moreover, biomass yields are boosted when diverse algal communities are grown together, which is thought to result from resource complementarity e.g. maximal use of light (Kazamia et al., 2014).

Synthetic ecology has been employed in microbial processes with beneficial environmental applications. For example, synthetic communities have been constructed for enhanced performance in microbial fuel cells for increased energy generation. In such studies, electrogenic species (e.g. *Geobacter*, *Enterobacter aerogenes*, *Shewanella oneidensis*) were co-cultured with non-electrogenic bacterial species (e.g. *E. coli*, *Pseudomonas aeruginosa*, *Lactococcus lactis*). The increase in power generation (compared to monocultures) is achieved by modification of the environment by the non-electrogenic species via removal of dissolved oxygen (Qu et al., 2012) and/or removal of fermentation by-products (Bourdakos, Marsili, & Mahadevan, 2014; Venkataraman et al., 2011), which makes conditions more favourable for the growth of the electrogens. The non-electrogenic species also provide the electrogenic species with substrates (Rosenbaum et al., 2011) or electron mediators (Venkataraman et al., 2011). Synthetic communities have also been used for the bioremediation of various pollutants. As well as the aforementioned example of a synthetic community used for the degradation of 4-chlorosalicyclate (see above), synthetic communities have also been used for the bioremediation of pesticides (Li et al., 2008), metals and dyes (Mishra & Malik, 2014). In the study by Li et al., *E. coli* was genetically engineered to over-produce methyl parathion hydrolase, and was grown in co-culture with an *Ochrobactrum* strain that utilized p-nitrophenol, a product of methyl parathion hydrolysis (Li et al., 2008). A co-culture of *Aspergillus lentulus*, *A. terreus* and *Rhizopus aryzae* was also shown to remove Cu^{2+}, Cr^{6+} and dyes better than their monoculture counterparts (Mishra & Malik, 2014). Removal of biofouling by synthetic communities of genetically engineered *E. coli* has also been demonstrated. These biofilms are able to displace existing biofilms, but are then removed on command with a chemically induced switch (Hong et al., 2012).

Finally, synthetic ecology has potential applications in human health, in particular, where host function relies on microbiota. In a pioneering study, Lawley et al. showed that a taxonomically-diverse mix of 6 cultured species isolated from healthy mouse faeces could restore *Clostridium difficile*-associated dysbiotic mouse GI tracts to normal levels of diversity and taxonomic composition (Lawley et al., 2012). In human patients, Petrof et al. demonstrated that diarrhoea

caused by CDI could be eradicated upon transplant of a synthetic community of 33 gut isolates from a healthy donor into the right and mid colon (Petrof et al., 2013). This therapy was implemented after patients were unresponsive to treatment with metronidazole or vancomycin, demonstrating its potential use in combatting infectious diseases without the use of antibiotics. Importantly, though, the target GI tract in the recipient host is an open system, in which naturally-acquired microorganisms can supplement those supplied in the artificial bacteriotherapy mix, leading to a final community state far more taxonomically- and functionally-diverse than could easily be assembled in the laboratory. In fully closed systems, the assembly and maintenance of stable and complex communities remains problematic and illustrates our incomplete knowledge of how these communities are maintained in nature.

5.2 Artificial Ecosystem Selection

Artificial ecosystem selection (AES) works by selecting successive generations of ecosystem "units" based on their "phenotypic trait" (i.e. ecosystem function), much like selective breeding for individual organisms. It is therefore a "top-down" approach to manipulating diversity to direct overall ecosystem function. The selected phenotype may be some physical trait (e.g. pH, redox potential), or its ability to carry out a process (e.g. degradation of a pollutant, plant fitness, flask performance). Each "off-spring" community is created through inoculation of a sterile neutral environment with microbes obtained from the "parent" community, and over time, the ecosystem improves on its selected trait. Multiple off-spring communities can be made from a single parent, but only those that show improvement of the trait will be selected as parents for subsequent generations.

This creation of "designer ecosystems" approach allows selection of function without prior knowledge of the roles of individuals, thereby circumventing the laborious and time-consuming task of cultivating and isolating individuals of interest, followed by extensive genomic and functional analyses. Since existing relationships and interactions are also present, it circumvents the need to test combinations of individuals for a functioning ecosystem, which is often difficult to achieve.

The earliest examples of artificial ecosystem selection come from work done by Swenson *et al*. In a terrestrial ecosystem, high and low plant biomass production was selected, and in an aquatic ecosystem, high and low pH, was selected. In both these ecosystems, there was a significant divergence in phenotypic traits (i.e. high vs low) (Swenson, Wilson, & Elias, 2000). In a further experiment, aquatic ecosystems were used to select for the ability to degrade the toxic compound 3-chloroaniline. Over 30 generations, three of the four lines increased in their ability to degrade the toxin compared to non-selected lines i.e. parents selected regardless of performance (Swenson, Wilson, & Elias, 2000; Swenson, Arendt, & Wilson, 2000).

The concept of artificial ecosystem selection was later picked up by others. In a computer simulation designed to mimic the growth of evolving microbial communities in flasks, liquid medium was seeded with a simulated microbial inoculum consisting of "species" with different nutrient and environmental preferences, and high and low ecosystem performances were selected. A control line was also simulated where no selection was imposed. Interestingly, in this simulation, convergence of all three lines is observed after selection for performance is ceased (after 30 generations), and flasks are instead chosen at random (Williams & Lenton, 2007). This modelling study supports the hypothesis that heritability of a phenotypic trait in artificial ecosystem selection acts at the community level, ruling out the possibility of selection at the individual species level where a single species is concurrently selected giving the appearance of heritability, while other species are irrelevant to the response (Williams & Lenton, 2007).

In the Winogradsky column system, the large variation in diversity produced in replicate microcosms can be circumvented if the communities are pre-conditioned to the microcosm environment, making it easier to predict the final microbial diversity (Fig. 7) (Pagaling et al., 2014). Replicate sterile microcosms were inoculated with material from two different mature Winogradsky columns ("parents"), with significant differences in their microbial composition. The resulting "daughter" microcosms develop more taxonomically-predictable communities that are closely related to those of the "parent" columns. This is most probably due to the reduced amplification of rare species and pre-adaptation to the selective bottlenecks during microcosm development. This finding has implications for systems that rely on microbial processes (e.g. wastewater treatment), since pre-conditioned microbial communities would have more predictable ecosystem functions, making such systems less likely to fail. However,

multi-generational selection for a particular property is more difficult in a system like the Winogradsky column, due to the complex and integrated functions of the diverse community.

Complex systems theory was used to explain variability at the ecosystem level (Swenson, Wilson, & Elias, 2000). Under this theory, replicate complex systems containing arbitrarily small initial differences may produce vastly different systems over time (e.g. the "butterfly effect" in weather systems). That is why in the experimental systems, identical physical conditions inoculated with seemingly identical inocula (or at least inocula with extremely small differences as it contains millions of microorganisms taken from the same source) produced vastly different off-spring with respect to performance of the selected ecosystem function.

However, if the ecosystems were dependent on the initial conditions, there would be no heritability. Therefore, local stability of combinations of species (and the genetic composition they carry) is required for their properties to be inherited by their offspring (Swenson, Wilson, & Elias, 2000). This has both positive and negative effects on ecosystem selection as was demonstrated by the toxin degradation experiment. One of the four selected lines for increased toxin degradation actually showed *decreased* performance over time. Similarly, one of the non-selected lines showed increased performance over time, despite the absence of selection. Both lines happened to be extremely stable with respect to their ability (or not) to degrade the toxin (Swenson, Arendt, & Wilson, 2000). Nevertheless, in selected lines, this property allows stable combinations of species to prevail over less stable ones, and appears as heritability of phenotypic traits in response to selection.

Most recently, this concept has been used to modify host-associated microbiota, selecting for microbial ecosystems that show improved function in the host that is biologically, medically or economically important, such as biomass production, fecundity, pathogen resistance or environmental (e.g. drought) tolerance (Mueller & Sachs, 2015). In this method, the host is used as an indicator of ecosystem function. Taxonomically distinct microbial populations within the rhizosphere were successfully selected to influence plant flowering times, such that one rhizosphere community induced early flowering times, while the other induced late flowering times. Interestingly, transfer of these communities onto novel plant hosts also produced consistent responses with respect to flowering times, depending on host genetics (Panke-Buisse et al., 2015). In an experiment selecting for drought tolerant plants, seeds were

propagated from the best performing plants, but no selection was imposed on the soil microbial communities. Instead, soil was perpetuated throughout the experiment; however, microbial communities changed nevertheless due to plant-microbiome interactions and adaptation to soil moisture conditions. This was supported by analysis of the wet and dry adapted soil microbial communities, which were found to be taxonomically distinct. Increased plant fitness was observed when they were associated with microbial communities adapted to wet or dry conditions (Lau & Lennon, 2012). In both these studies, the observed host traits were postulated to be a result of modification of the soil environment by the selected microbial communities. These studies demonstrate that below-ground adaptation has a stronger effect than evolutionary changes in the plant populations (Lau & Lennon, 2012; Panke-Buisse et al., 2015).

5.3 Bioaugmentation for Engineering of Specific Community Functions

Bioaugmentation is the addition of biological material to improve ecosystem function. This concept is not new; humans have been relying on this technique for years for the preparation of beer, bread, cheese and yoghurt, which involves inoculation with a starter culture. These days, bioaugmentation is being used as a strategy for improving bioremediation (Lebeau, Braud, & Jezequel, 2008; Tyagi, da Fonseca, & de Carvalho, 2011; Cycon, Mrozik, & Piotrowska-Seget, 2017) and wastewater treatment (Herrero & Stuckey, 2015) where bioaugmentation is advantageous as the contaminants need to be treated *in situ*. However, it is also being used for industrial processes, such as improving gaseous biofuel production, (e.g. (Acs et al., 2015). In this review, we will only consider the use of microorganisms for bioaugmentation strategies, but the addition of plants, genes and enzymes have also been used (Zouboulis, Loukidou, & Christodoulou, 2001; Top, Springael, & Boon, 2002; Pilon-Smits, 2005; Cycon, Mrozik, & Piotrowska-Seget, 2017).

Depending on the application, the microbial community composition of the inoculum does not need to be known. In the aforementioned example of bread making, sourdough starter cultures are often made using wild yeast and bacteria from the air and surfaces, and subsequent batches of sourdough bread are made by perpetuating this starter culture through feeding with fresh flour and water. Similarly, this approach can be taken for other bioaugmentation strategies. For example, the start-up and stability of a pilot-scale partial nitrification reactor involved in

wastewater treatment was greatly improved upon seeding with an activated sludge inoculum from a pilot plant performing nitrification under stable conditions compared to the reactor that was seeded with conventional activated sludge (Bartroli, Carrera, & Perez, 2011). In two other studies, pentachlorophenol (PCP) contaminated soils bioaugmented with "activated" soil (i.e. soil that was pre-conditioned to degrade PCP) (Barbeau et al., 1997) or with a PCP-degrading methanogenic consortium (Beaudet et al., 1998) showed higher rates of PCP degradation compared to non-bioaugmented soils. In all these cases, the microbial inoculum was pre-adapted, and so the function of the microbial community was known, but the exact microbial community composition was not. The addition of complex microbial consortia, by increasing biodiversity, increases the genetic potential of the community and thus can contribute positively to overall ecosystem function and bioremediation efficiency (Dejonghe et al., 2001).

In most applications, however, the inocula are composed of known microorganisms with a specific function to allow targeted improvement of ecosystem function. There are numerous examples of single microbial species used to bioaugment a variety of processes, such as the bioremediation of pesticides from soil (Cycon, Mrozik, & Piotrowska-Seget, 2017) and the degradation of chemicals from wastewater (Herrero & Stuckey, 2015). In the same study in which an uncharacterised microbial consortium was used to enhance biodegradation of PCP, a single additional PCP-degrading organism, *Desulfitobacterium frappieri* (now *Desulfitobacterium hafniense*) strain PCP-1, was shown to degrade the pollutant effectively in a soil community context (Beaudet et al., 1998). However, the added strain does not persist for long periods of time within the soil community and, in the presence of high levels of PCP, this results in incomplete degradation unless multiple bioaugmentations over time are carried out. This illustrates the difficulty of ensuring persistence of an exogenous strain in an already assembled and stable community. Alternatively, organisms added to a system for a particular bioremediation purpose may be overshadowed by rare endogenous species selected by the target contaminant. In a study of hydrocarbon-supplemented soil microcosms, Fuentes *et al*. (Fuentes et al., 2016) found that a bloom of a previously-rare *Alkanindiges* species reached far higher abundance in the presence of diesel oil than a defined bacterial consortium or an enrichment added specifically to boost oil degradation. Despite this, the bioaugmented organisms were maintained in the community over 6 weeks and contributed to oil degradation, showing that community persistence following bioaugmentation, while poorly understood, is possible.

For complex microbial processes, such as the degradation of biomass or chemical mixtures, a combination of species may be used. Similar to synthetic ecology (see previous section), the microbial inoculum may contain microorganisms with complementary metabolic functions to allow cooperative relationships to form. For example, a mixed fungal-bacterial culture, in which either a single bacterial *Stenotrophomonas* species or a bacterial consortium is combined with the fungus *Penicillium janthinellum*, is able to degrade polycyclic aromatic hydrocarbons (PAHs) when added to PAH-contaminated soil (Boonchan, Britz, & Stanley, 2000). However, some members of the inoculum may be used to enhance the performance of others. In a study by Jacques et al, a microbial consortium of naphthalene-degrading bacteria, namely *Mycobacterium fortuitum*, *Bacillus cereus*, *Microbacterium* sp., *Gordonia polyisoprenivorans*, and a *Microbacteriaceae* bacterium, and the fungus *Fusarium oxysporum* were used to treat PAHs-contaminated soil. The addition of the microbial consortium was shown to be essential for PAH degradation as the addition of individual species did not degrade the PAHs. It was hypothesised that this is due to the presence of the fungus, as fungal hyphae can act as dispersion vectors for bacteria in soil (Jacques et al., 2008).

In conclusion, different approaches to microbial community manipulation are becoming available to applied microbial ecologists as our characterisation and understanding of the structure and function of natural communities increases. While synthetic ecology approaches may seem to offer the clearest route to producing bespoke microbial communities for particular purposes, to date our ability to construct stable, defined communities, even using well-understood microbial strains, has been limited to simple combinations orders of magnitude less complex than natural systems. This may in part be due to the factorial expansion of possible inter-species interactions as complexity increases: assuming 6 possible modes of interaction between any pair of species, a simple 3-species system has 729 possible interaction states, while for a 4-species system, the number of combinations is 531,441 (Großkopf & Soyer, 2014). This level of complexity probably also underlies the potential difficulties in achieving stable bioaugmentation of established, stable communities with defined species or consortia. One potential route to overcoming the latter problem may be inoculation when the system is in a state of disturbance, allowing the bioaugmented species to become integrated as the interaction network re-establishes itself. Such disturbance is, however, easier to achieve in a bioreactor or even a human GI tract (via colonic washout (Khoruts et al., 2010)) than in the natural environment. AES approaches have currently not been developed widely, but the recent resurgence in interest in them promises more conclusive tests in a variety of systems. The

combination of all these approaches, for instance via bioaugmentation with a simple consortium defined by laboratory-based synthetic ecology experiments, followed by ecosystem-level selection of the bioaugmented community for the final desired function, may offer the greatest potential for reliable in any real-world (i.e. open system) context.

6. FUTURE PERSPECTIVES

Our understanding of the scale of microbial diversity has advanced enormously in recent years, due not just to the ease of in-depth characterization of individual environments, but also to the careful application of theoretical approaches to constrain data (Curtis, Sloan, & Scannell, 2002; Curtis & Sloan, 2004). Lagging somewhat behind this understanding is an ability to map taxonomic variation to functional properties, due to a combination of the still vast number of uncharacterized genomes (microbial dark matter), the massive complexity of the interaction networks between these myriad species and the rapid and diverse means by which microorganisms can evolve on timescales comparable to those of ecological approaches. Nevertheless, combining carefully designed experiments aimed at elucidating general theories and principles (Prosser et al., 2007) with appropriately tractable and relevant microbial systems offers a way to make efficient progress through this "morass of diversity" (Prosser, 2012). Microbial microcosms such as those described in this review, along with well-understood systems in the biotechnological (e.g. anaerobic digestion and wastewater treatment) and medical (e.g. the human GI tract) fields, offer appropriate opportunities for developing and testing general theories and principles. Although not discussed in detail here, appropriate mathematical models also have a crucial role to play in developing hypotheses based on experimental data, testing them *in silico* and then suggesting further experimental tests or optimization strategies (Widder et al., 2016; Narayanasamy et al., 2015). Moving away from the "stamp-collecting" phase of microbial community characterization to these more focused hypothesis-testing approaches offers vast opportunities for applied microbial ecology in the coming years.

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Table 1 Current methods used in the characterisation of microbial diversity and function

Method	Description	Example
Microbial diversity (who is there?)		
Metataxonomics	Study of a single conserved gene, usually the ribosomal RNA (rRNA) gene.	(Pagaling et al., 2017)
Microbial community's functional potential (what can they do?)		
Metagenomics	Study of all the genomes present in a given environment	(Tringe et al., 2005)
Functional gene assays	Monitoring of functional genes to target microorganisms involved in a particular function e.g. nitrogen cycle	(Dandie et al,. 2011)
Microbial gene expression (what are they doing?)		
Metatranscriptomics	Study of the RNA transcribed in a given environment to determine the metabolically active portion of the microbial community	(Jiang et al., 2016)
Metaproteomics	Study of the proteins expressed in a given environment either over time and/or in association with a specific treatment.	(Zhang et al., 2017b)
Metametabolomics	Study of naturally-occurring, low molecular weight organic metabolites to characterise the interactions of living organisms with their environment and/or other organisms.	(Yang et al., 2011)
Functional characterisation (what are the ecological consequences?)		
Novel isolation methods	Utilisation of cultivation methods which match the native nutritional requirements for microorganisms and prevent over-reliance on chemically synthetic medium.	(Nichols et al,. 2010)
Genome reconstr-uction from metagenomes	Utilisation of genome sequences to elucidate function of microorganisms which have proved resistant to culture.	(Carr et al., 2018)

BOX 1

Can general ecological theories be applied to microbial communities?

For decades, macroecologists have utilized general theories to explain patterns observed in biodiversity, species abundance and species richness. Recently, there has been an upsurge of interest in examining whether these theories apply to life at the microbial scale (Prosser et al., 2007). Here we discuss several of these theories and present findings on how they govern dispersal and distribution of microorganisms within the biosphere.

1. **The Species-Area Relationship**

One of the oldest theories in ecology explores the relationship between the size of a habitat and the number of species present. This has been routinely observed for macroorganisms, and the work of Horner-Devine et al. (2004) suggests that a similar taxa-area relationship exists for bacteria, whereby more similar bacterial communities are found closer to each other within salt marsh sediments, and communities differ as distance increases. Furthermore, Bell et al. (2005) showed that larger isolated biological "islands" (rain-filled tree hollows, deriving energy from the degradation of plant material) contained a higher number of bacterial taxa than smaller islands, suggesting that habitat size strongly correlates with higher diversity with bacterial populations in stable and relatively isolated environments.

2. **Productivity-Diversity Relationships**

The relationship between species diversity and ecosystem productivity (the rate of conversion of energy and abiotic factors into biomass) is deeply rooted in the traditional ecological literature. However, studies have revealed a similar relationship in microbial systems. Horner-Devine et al. (2003) observed that primary productivity could influence species richness in freshwater mesocosms. The species richness within the *Cytophaga-Flavobacteria-Bacteriodes* group showed a significant hump-shaped relationship (showing maximum species diversity at levels of intermediate primary productivity), as is often observed for marine and freshwater plants and animals. Likewise, species manipulation in the tree-hollow microcosm system should a positive, though decelerating, effect of increased species richness on community respiration (Bell, Newman, et al., 2005). Importantly, the taxonomic composition of the community and the presence of synergistic interactions between its individual members also had a significant role in determining productivity.

3. **The *"Everything is Everywhere"* Hypothesis**

In the early 20th century, the Dutch microbiologist Martinus Wilhelm Beijerinck developed his hypothesis that microbial distribution within the biosphere was determined by the environment. This led to Lourens Baas-Becking's well cited principal that "everything is everywhere but the environment selects" which is continually reported in both microbial and theoretical ecological literature (de Wit & Bouvier, 2006). Baas-Becking's use of enrichment cultures revealed the growth of latent or low-abundance microorganisms, and therefore this work led to the belief that all microorganisms are ubiquitous and show a cosmopolitan lifestyle but cannot always be observed as may be present in low numbers (i.e. "everything is everywhere"). Evidence for the claim remains unclear. More recently, microbiologists are aware of the challenges involved in the cultivation of microbial dark matter and the presence of rare species, which has only added to the complexities of Baas-Becking's original statement. Furthermore, detection of rare taxa

becomes difficult due to threshold limitations which impacts any attempt to solidify that fact that “everything is everywhere”.

However, many works have suggested that environmental conditions do explain apparent geographical differences observed in microbial communities, such as soils, salt marshes and the microbiota of corals (O'Malley, 2007; Horner-Devine et al., 2004; Horner-Devine, Carney, & Bohannan, 2004). The fact that microbial communities may show biogeography, and community similarity across large geographical distances (influenced by abiotic and biotic environmental factors) may lead to a greater understanding of how microorganisms assemble within communities. The question of whether a truly universal dispersal of the microbial diversity of the biosphere is possible, allowing predictable environmental selection of the best-adapted organisms from the global species pool, remains technically open but seems unlikely in the face of our present-day understanding of that diversity.

4. Resilience and Redundancy of Microbial Ecosystems.

Microbial community **resilience** refers to the consortium’s ability to recover after community perturbation has occurred. If community function is linked to a stable community structure, it could be predicted that the function of the community may be impacted negatively due to the loss of key microbial species during community disruption. A meta-analysis of studies of disturbance in microbial ecosystems suggests that contrary to common perceptions, resilience to perturbation is not generally observed (Allison & Martiny, 2008). However, other studies suggest that some natural communities may exhibit greater resilience (Shade et al., 2012b), and resilience to environmental challenge has been observed in both large- and small-scale anaerobic bioreactors (Werner et al., 2011; Fernández et al., 2000).

Microbial **redundancy** is the ability of different microorganisms to substitute each others’ metabolic roles (e.g. two or more species acting as cellulose degraders). Shade et al. (2012a) argue that functions performed by many phylogenetically distinct taxa in a community (therefore, a higher level of functional redundancy) will recover more easily from disruption and the ecosystem services provided may not suffer. Conversely, if a specific process is only carried out by rare, low abundance species, the ability for other microorganisms to show redundancy will be low, and therefore the function may cease following community breakdown.

FIGURE LEGENDS

Figure 1 Access to abundant and rare microbial community members with molecular and mixed culture-based techniques. Microbial communities (*dashed circle*) contain a core of abundant species (*red-orange*) and a large number of much rarer species (*yellow-white*). Different molecular techniques (clone libraries, fingerprinting techniques and next-generation metataxonomic sequencing) can access this species distribution to different depths (*black wedges*). Mixed culture techniques (microcosms, bioreactors) select species from all parts of the species distribution depending on their ability to proliferate in the environmental conditions of the reactor.

Figure 2 Aspects of microbial community structure and function described by molecular techniques. DNA extracts can be used for metataxonomics of marker genes (normally 16S rRNA genes) or for full metagenome analysis (community genetic potential). RNA and protein extracts can be used for metatranscriptomics or metaproteomics to determine gene expression and its protein products. Metametabolomics documents the biochemical consequences of this expression of potential function at the community level.

Figure 3 Pathways of biogas production in anaerobic digestion systems. Feedstock compounds, intermediates and breakdown products (*blue boxes*) are interconverted by specific functional groups of microorganisms (*red boxes*) via the four main processes of anaerobic digestion (*capitals*) to produce biogas, a mixture of methane and carbon dioxide.

Figure 4 Important chemical reactions in anaerobic digestion. Acetogenesis occurs via the reductive H_2/CO_2 pathway (*A*) or from longer-chain fatty acids in energetically-unfavourable reactions (*B*). These can be coupled syntrophically to hydrogenotrophic methanogenesis (*C*), which also competes for substrates with acetogens performing reaction *A*. Acetaoclastic methanogenesis also converts acetate directly to biogas (*D*). Free energies (ΔG) of the reactions under standard conditions are shown.

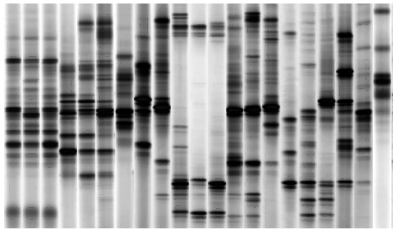
Figure 5 Vertical layering and carbon- and sulphur-cycling in Winogradsky column microcosms. (*A*) A vertical layered structure with opposing gradients of oxygen and sulphide forms in mature microcosms. Functional groups dominating each layer are listed (*right*) along

with illustrative taxa. (*B*) Microbial functional groups and intermediates involved in the carbon (*black*) and sulphur (*red*) cycles in microcosms. Anaerobic methanotrophic *Archaea* (*ANME*) carry out anaerobic methane oxidation in syntrophy (*brackets*) with sulphate-reducing *Bacteria*.

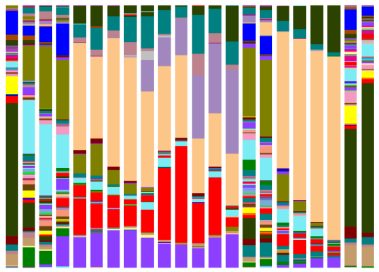
Figure 6 Selection bottlenecks, community diversity and variability during microcosm development. Initial species selection following transfer into the microcosm (*1*) is followed by further selection as *Firmicutes* proliferate, cellulose is degraded and sulphide is produced (*2*). Following this second bottleneck, the community re-diversifies and microcosms diverge into *Firmicutes*- and *Bacteroidetes*-rich sub-populations (*3*). The ratio of these to each other is affected by system size (*lower right*).

Figure 7 Microcosm community predictability depends on the source community. (*A*) Communities from three environmental sources become less similar to each other when inoculated into the common microcosm environment, although a compositional signal from the source is maintained. (*B*) When mature microcosm communities are used as source inocula for replicate microcosms, the resulting microcosm communities are highly reproducible and related to the source community.

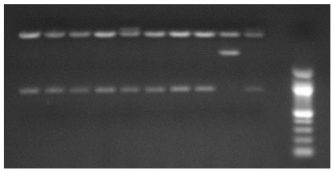
Fingerprinting



Metataxonomic Sequencing



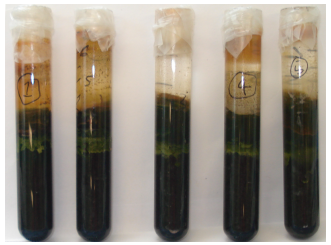
Microbial Community



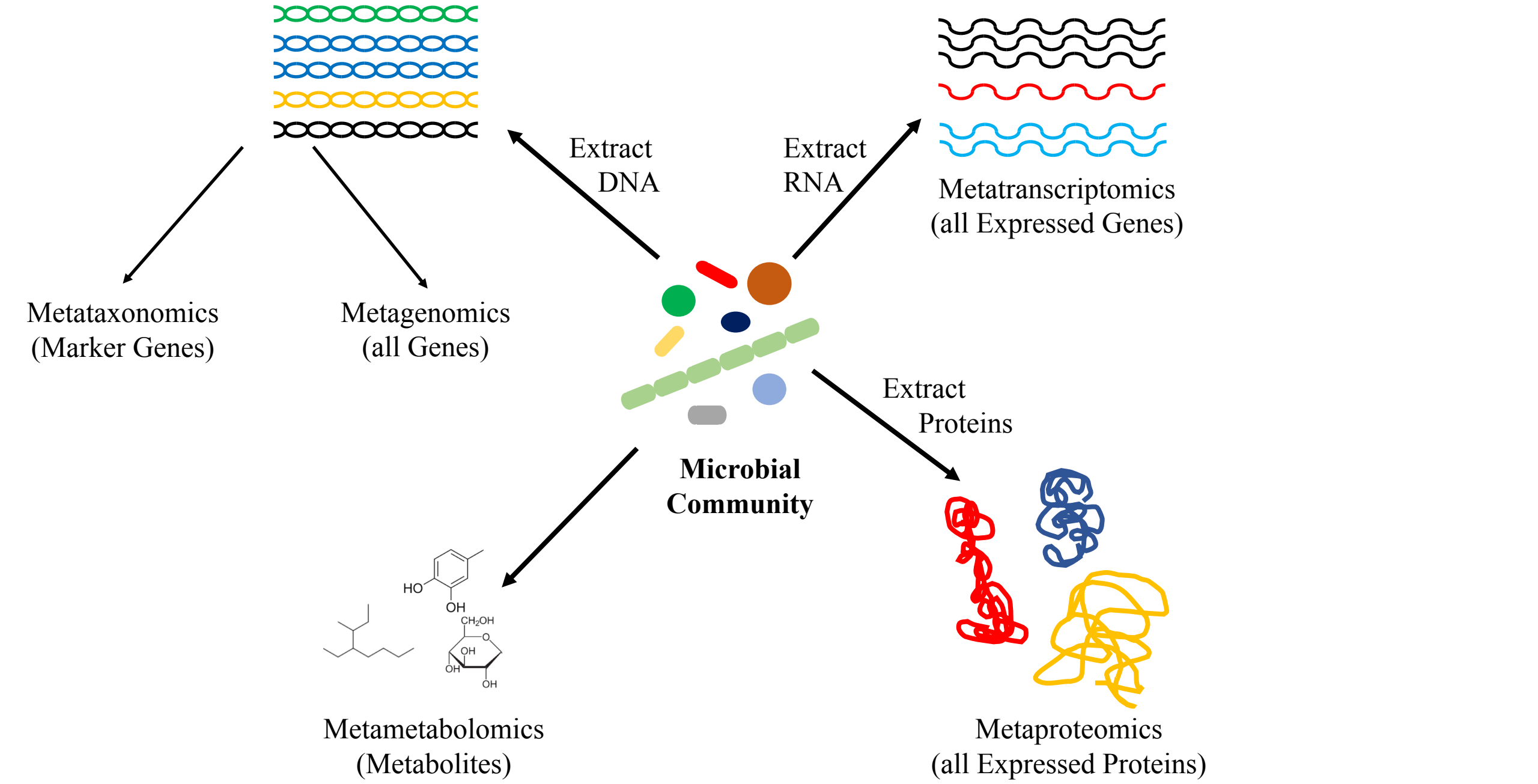
Clone Libraries

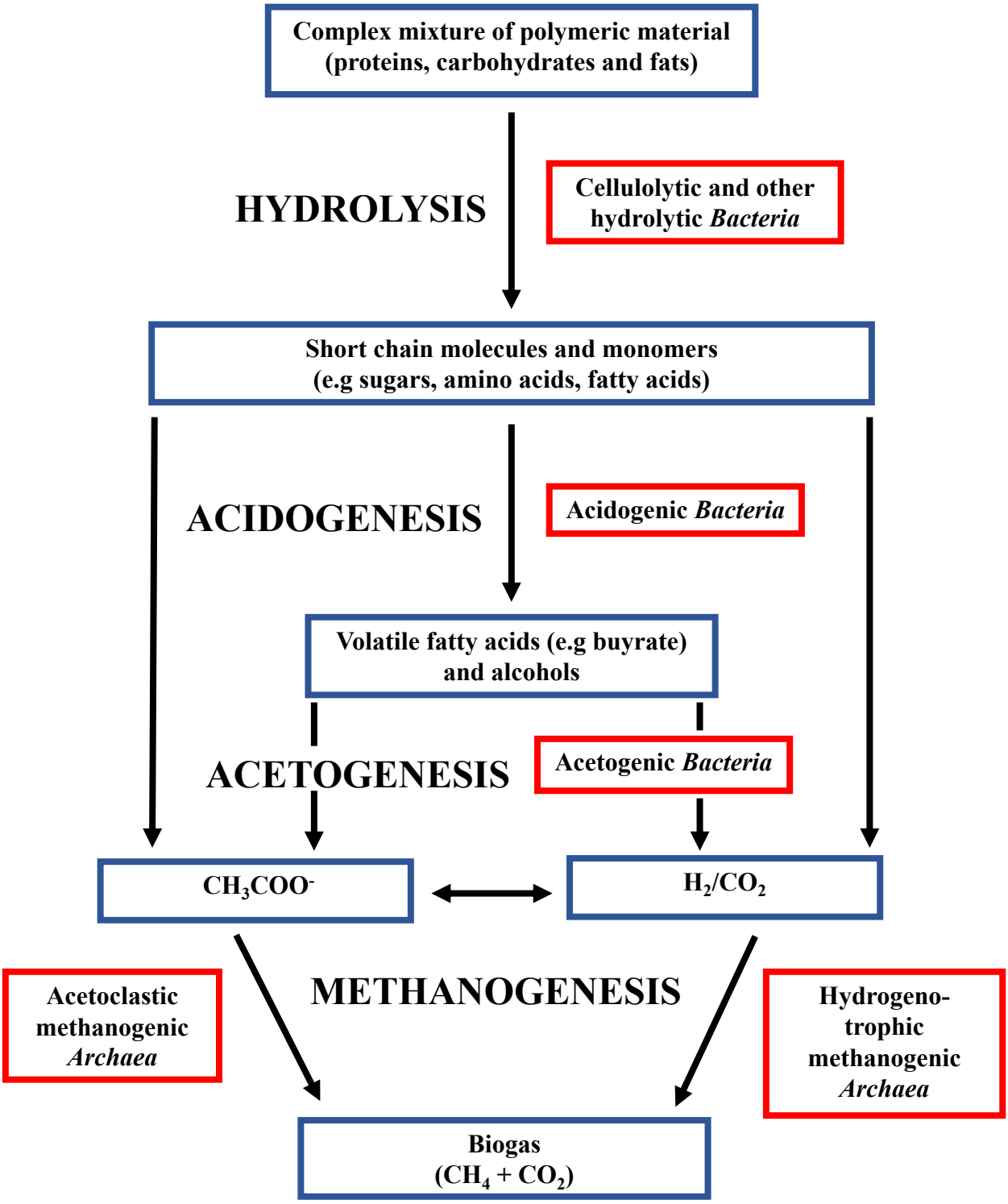
Abundant

Rare



Microcosms & Bioreactors


Extract
DNA
Extract
RNA
Metatranscriptomics
(all Expressed Genes)
Metataxonomics
(Marker Genes)
Metagenomics
(all Genes)
Extract
Proteins
Microbial
Community
HO
OH
CH2OH
O
OH
OH
OH
Metametabolomics
(Metabolites)
Metaproteomics
(all Expressed Proteins)


Complex mixture of polymeric material (proteins, carbohydrates and fats)
HYDROLYSIS
Cellulolytic and other hydrolytic *Bacteria*
Short chain molecules and monomers (e.g sugars, amino acids, fatty acids)
ACIDOGENESIS
Acidogenic *Bacteria*
Volatile fatty acids (e.g buyrate) and alcohols
ACETOGENESIS
Acetogenic *Bacteria*
CH_3COO^-
H_2/CO_2
METHANOGENESIS
Acetoclastic methanogenic *Archaea*
Hydrogeno-trophic methanogenic *Archaea*
Biogas ($CH_4 + CO_2$)

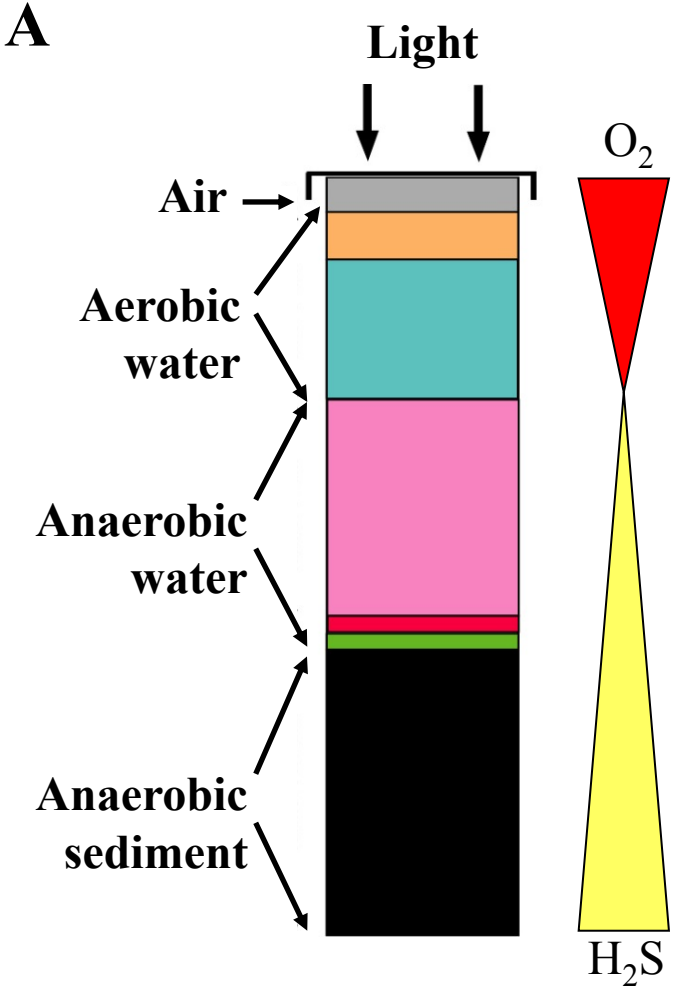
A) $2CO_2 + 4H_2 \rightarrow CH_3COO^- + H^+ + 2H_2O$ ($\Delta G^{o\prime} = -95$ kJ/mol)

B) $C_4H_7O_2^- + 2H_2O \rightarrow 2CH_3COO^- + H^+ + 2H_2$ ($\Delta G^{o\prime} = +48.3$ kJ/mol)

C) $CO_2 + 4H_2 \rightarrow CH_4 + 2H_2O$ ($\Delta G^{o\prime} = -131$ kJ/mol)

D) $CH_3COO^- + H^+ \rightarrow CH_4 + CO_2$ ($\Delta G^{o\prime} = -36$ kJ/mol)

A



Predominant Organisms

Cyanobacteria, algae

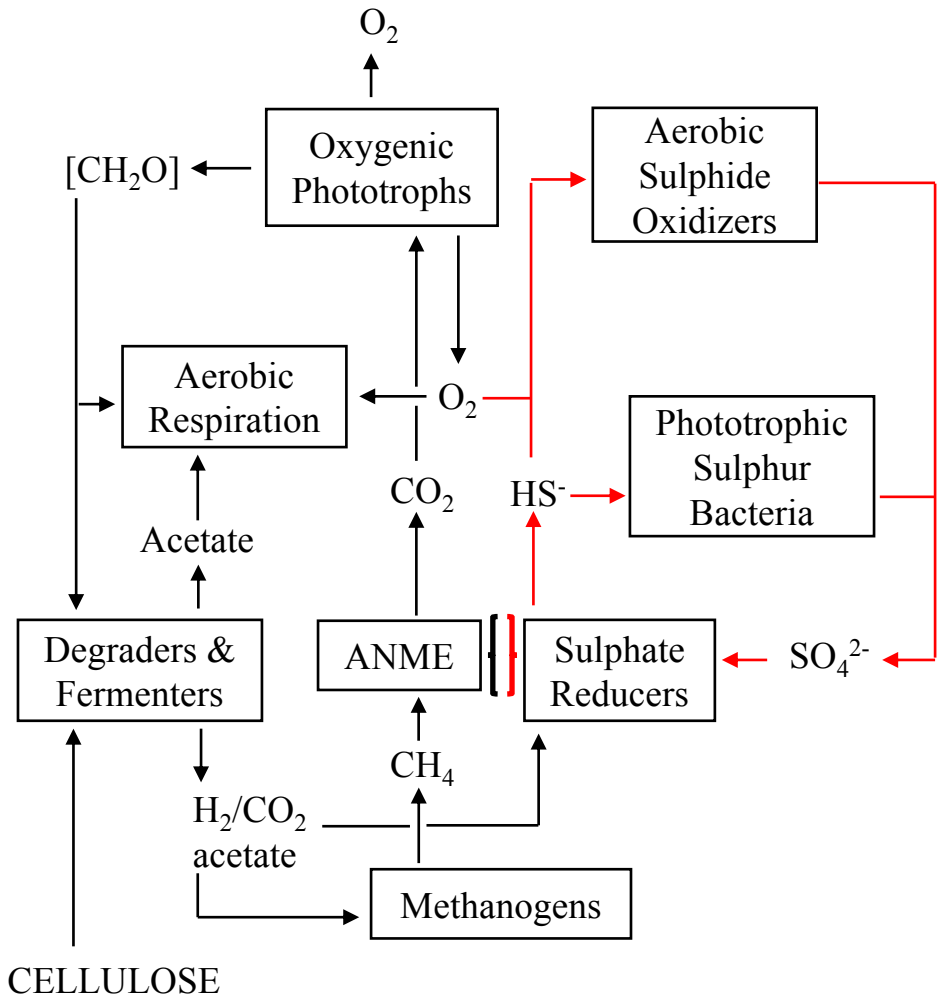
Purple non-sulphur bacteria (e.g. *Rhodospirillales*)

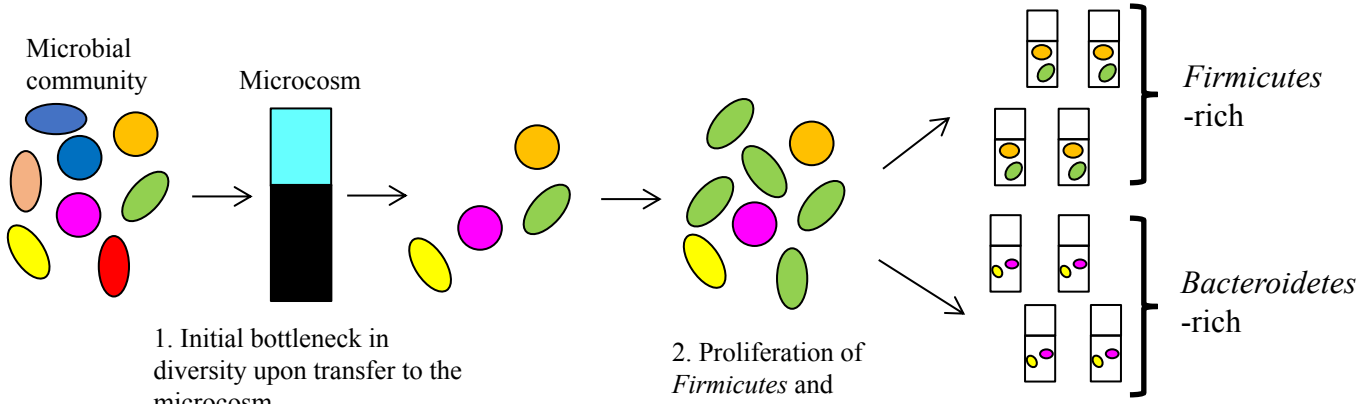
Purple sulphur bacteria (e.g. *Chromatiales*)

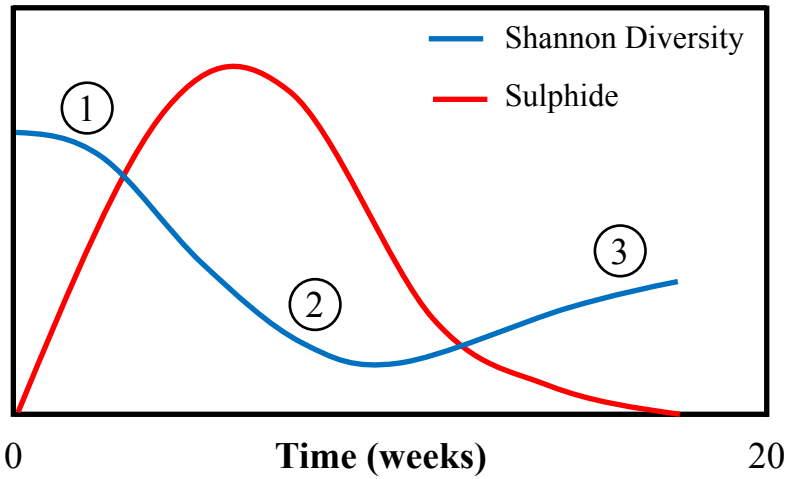
Green sulphur bacteria (e.g. *Chlorobiaceae*)

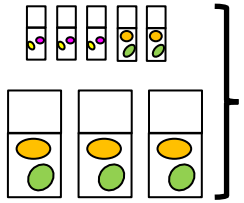
Anaerobic heterotrophic decomposers (*Firmicutes*, *Bacteroidetes*), methanogens and sulphate reducing bacteria (e.g. *Desulfovibrio*)

B



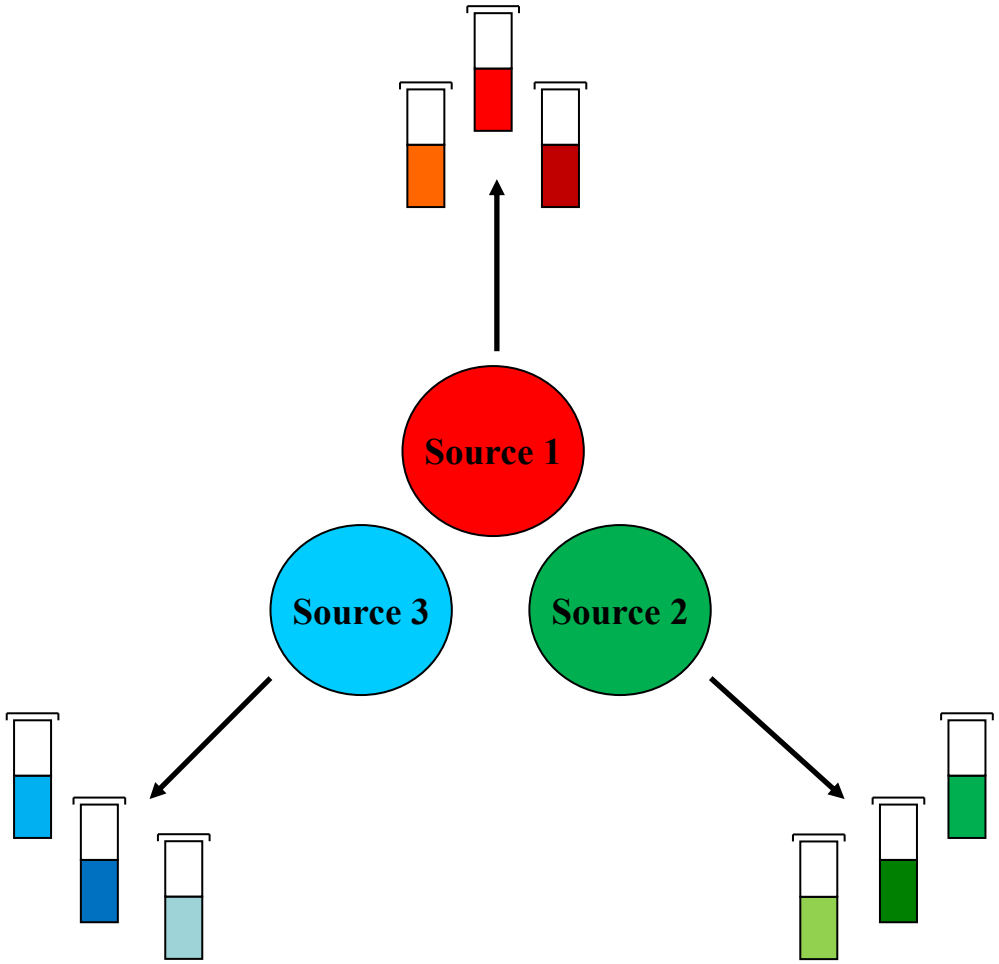






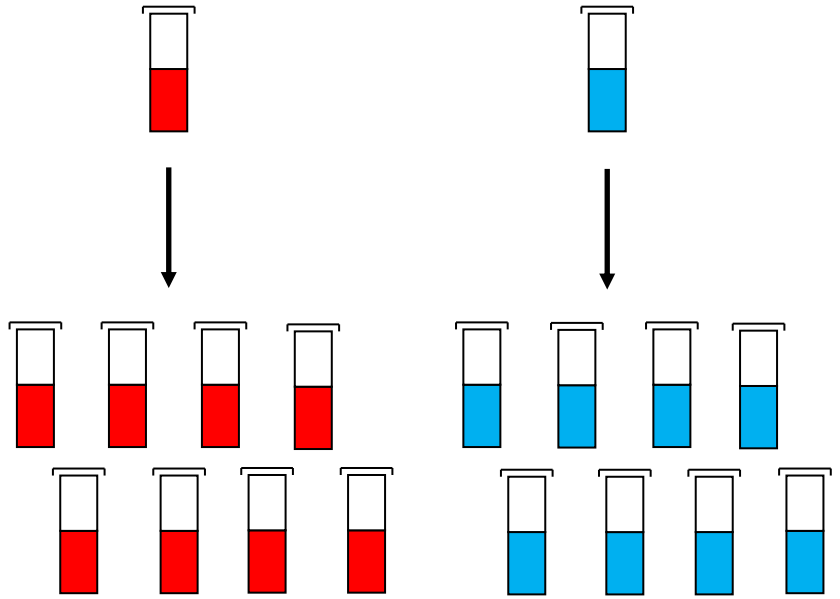
System sixe effect: larger microcosms consistently contain *Firmicutes*-dominated communities

A



Community composition is unpredictable
using environmental inocula

B



Community composition is predictable
using inocula pre-selected in microcosms